Phosphorus effects on arbuscular mycorrhizal fungi

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Abstract

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Phosphorus effects on arbuscular mycorrhizal fungi

Two field studies were conducted to assess the potential benefit of arbuscular mycorrhizal (AM) inoculation of elite strawberry plants on plant multiplication, and fruit yield, under typical nursery conditions, in particular soils classified as excessively rich in P. To study plant productivity, five commercially *in vitro* propagated elite strawberry cultivars ('Chambly', 'Glooscap', 'Joliette', 'Kent', and 'Sweet Charlie') were not inoculated with AM fungi or were inoculated with either a single species (*Glomus intraradices*), or a mixture of species (*G. intraradices*, *Glomus mosseae*, and *Glomus etunicatum*). AM inoculation was found to impact strawberry plant productivity in a soil with excessive P levels. The AM fungi introduced into the field by inoculated mother plants established a mycelial network in the soil through colonization of the daughter plant roots, however, persistence of colonization was determined to be low (<12 % in inoculated plant roots). In soils excessively rich in P, individual crop inoculation may be the only option for management of the symbiosis, as the host and non-host rotation crops, planted prior to strawberry production, had no effect on plant productivity or soil mycorrhizal potential.

To study the impact of AM inoculation on fruit production, three commercially grown strawberry cultivars (Glooscap, Joliette, and Kent) were not inoculated with AM fungi or were inoculated with either *G. intraradices* or *G. mosseae*. AM fungi impacted the fruit yield, with all inoculated cultivars producing more fruit than noninoculated cultivars during the first harvest year. The percentage of root colonization could not be used to explain the differences in total fruit yield during the first harvest year, or the increase in total fruit yield the second harvest year.

We wished to examine the effects of various P treatments on C metabolism within the intraradical mycelia (IRM) of the fungus. Specific primers were developed for the *Glomus intraradices* glucose-6-phosphate dehydrogenase (G6PDH) gene. Real-time quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) was used to measure the gene expression of the *G. intrarardices* G6PDH gene in response to external P conditions of colonized transformed carrot roots. The results showed a significant down-regulation of G6PDH in the IRM of *G. intraradices* when cultures were grown in a high P (350 μM P) medium compared to those grown in the low P (35 μM P) medium. The down-regulation may suggest a reduction in the C flow from the host to the fungus. There was no effect on G6PDH expression following a two-hour incubation with additional P applications (No P, low P and high P).

Résumé

Ph.D. Lynda Irene Stewart Microbiologie

Effet du phosphore sur les champignons mycorrhiziens à arbuscules

Deux expériences au champ ont été menées pour vérifier si l'inoculation de fraisiers élites avec des champignons mycorrhiziens (CM) à arbuscules peut influencer positivement la multiplication des plants et le rendement en fruits, en conditions typiques de production et plus particulièrement en sols classifiés excessivement riches en P.

Pour étudier la productivité des plantes, cinq cultivars ('Chambly', 'Glooscap', 'Joliette', 'Kent', et 'Sweet Charlie') de fraisier propagés *in vitro* selon un protocole industriel de production de plants elites n'ont pas été inoculés avec des CM, ou l'on été avec une espèce (*Glomus intraradices*), ou avec un mélange d'espèces (*G. intraradices*, *Glomus mosseae*, et *Glomus etunicatum*).

L'inoculation a eu un impact sur la productivité des fraisiers en sol excessivement riche en P. Les CM introduits au champ par inoculation des plants mères ont établient un réseau mycélien dans les sols et colonisé les racines des plants filles, même si la persistance de la colonisation était faible (<12 %) chez les plants inoculés. En sols excessivement riches en P, l'inoculation est peut-être la seule façon possible d'envisager la gestion de la symbiose, puisque la rotation de culture

avec plantes hôtes ou non-hôtes n'a pas eu d'effet sur la productivité des plants ou sur le potentiel mycorrhizien du sol.

Pour étudier l'impact de l'inoculation avec CM sur la production de fruits, trois cultivars de fraisiers (Glooscap, Joliette, et Kent) n'ont pas été inoculés avec CM ou ont été inoculés avec le *G. intraradices* ou le *G. mosseae.* Les CM ont influencés les rendements, tous les cultivars produisant plus de fruits que les plants non-inoculés, la première année de récolte. Le pourcentage de colonisation racinaire n'expliquait pas les différences des rendements en fruits pendant la première année de récolte ou l'augmentation de la productivité observée en deuxième année.

Les effets de différents niveaux de P sur le métabolisme du C dans le mycélium intraracinaire (MI) du champignon ont été déterminés. Des amorces spécifiques ont été développées pour le gène de la glucose-6-phosphate déhydrogénase (G6PDH) du *G. intraradices.* La réaction en chaîne de la polymérase en temps réel et transcriptase renversée (real-time quantitative reverse transcriptase polymerase chain reaction, QRT-PCR) a été utilisée pour mesurer l'expression du gène G6PDH de *G. intraradices* en réponse à des niveaux environnementaux du P auxquels des racines mycorrhizées de carottes transformées étaient exposées. Les résultats indiquent une répression significative de l'expression du G6PDH dans le MI de *G. intraradices* lorsque celui-ci est cultivé en milieu de culture riche en P (350 μM P), comparer à un milieu pauvre (35 μM P). Cette répression suggère une réduction du flux de C de la plante au CM. Aucun effet sur l'expression du G6PDH n'a été observé suite à une incubation de deux heures avec une application supplémentaire de P (P 0, P pauvre et P élevé).

Contributions to knowledge

1. Reliance on fertilizer can be reduced through the use of AM fungi in high fertility soils.

2. Determined that in high fertility soils, inoculation with AM fungi can both increase and decrease strawberry daughter plant production.

3. Determined that in high fertility soils, inoculation with AM fungi can increase strawberry fruit production during the first harvest season.

4. In high fertility soils, inoculation of crops is favored over AM fungal management through crop rotation systems.

5. Developed *Glomus intraradices* specific primer encoding the glucose-6-phosphate dehydrogenase (G6PDH) gene.

6. Monitored the expression of the G6PDH gene in *G. intraradices* through the use of real-time quantitative RT-PCR.

7. Determined that C metabolic pathways within the IRM of the AM fungus *G. intraradices* can be influenced through external P concentrations.

List of abbreviations

AA	Amino acid
AC	Acid phosphatase
ALP	Alkaline phosphatase
AM	Arbuscular mycorrhizal (fungi)
CHO	Carbohydrate
ERM	Extraradical mycelia
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
IRM	Intraradical mycelia
MD	Mycorrhizal dependency
NMR	Nuclear magnetic resonance
Pi	Inorganic phosphate
PolyP	Polyphosphate
PPGK	Polyphosphate glucokinase
PPK	Polyphosphate kinase
PPP	Pentose phosphate pathway
QRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
TAG	Triacylglycerides
TCA	Tricarboxylic acid cycle

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Contributions of authors

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Chapter 1. Literature review

1.1 Introduction

Arbuscular mycorrhizal (AM) fungi belong to the phylum Glomeromycota (Schussler *et al.* 2001). These fungi have formed obligatory symbioses with the roots of plants for over 400 million years (Remy *et al.* 1996). The association with plants is believed to have occurred at the time of plant establishment on land (Simon *et al.* 1993). Currently, it is estimated that over 80% of vascular plants can form a symbiotic relationship with AM fungi. The ubiquity of this symbiosis has led to the natural distribution of AM fungi in soils throughout the world, and one of the most complex and important symbiotic relationships in nature.

Soil nutrients and photosynthetically-reduced C are exchanged between the fungus and the host. Increased nutrient uptake leading to increased crop yields is most prevalent in soils with low nutrient availability. Conventional agricultural practices often dictate that soils become over-fertilized with time, leading to an accumulation of P in the soil (Zhang *et al.* 1995, 2004). While these conditions are not ideal for AM symbioses, the persistent nature of the fungi may still allow them to overcome any detrimental effects posed by soils with high fertility, while reducing the reliance on additional fertilizer applications. This literature review primarily focuses on the interactions of AM fungi, host plants and soil P.

1.2 AM symbiotic structure

AM fungi forming symbiotic relationships with plant roots, consist of two phases: the internal mycelia, namely the intraradical mycelia (IRM) and the external mycelia, or the extraradical mycelia (ERM). Intracellular mycelia and intracellular arbuscules form within the cortical cells of the host root. The arbuscules are specialized structures that invaginate the plasma membrane of the host cells. The plasma membrane/arbuscule interface is thought to be the site for nutrient exchange between the host and the fungal partner (Giovannetti and Sbrana 1998).

The ERM extend in the soil beyond the surface of the host root to act as an extension of the root. The primary function of the ERM is acquiring nutrients otherwise unavailable to the host through enhanced nutrient translocation and uptake. There is a correlation between increased P uptake and increased ERM length in the soil (Jakobsen *et al.* 1992b). The ERM also serve as an inoculum source for secondary colonization of plant roots (Friese and Allen 1991).

1.3 AM symbiosis benefits

It is well documented that stronger, healthier, higher-yielding plants are produced from AM-inoculated root systems as a result of increased nutrient acquisition (Bolgiano *et al.* 1983; Wacker *et al.* 1990; Johnson *et al.* 1992; Miller 2000). Nutrient uptake by AM fungi is increasingly important in soils of low fertility. It is important in particular for nutrients such as P, Cu and Zn, which have low mobility in the soil (Jakobsen *et al.* 1992b). How well the fungus functions to supply

nutrients to the host plant is vital to the symbiotic relationship due to the fact that the fungal metabolism is regulated by the metabolism of the host (Shachar-Hill *et al.* 1995).

Other benefits obtained from the association host and fungus should not be overlooked. Factors such as reduced levels of water stress (Augé 2001; Davies *et al.* 2002), and lower disease incidence (Dehne 1982; St-Arnaud *et al.* 1995) contribute to the overall health and yield of the plants. Phytohormones are also known to play a role in the development process of plants and increases in phytohormone production have been shown to occur prior to other fungal derived benefits (Allen *et al.* 1980; 1982; Shaul-Kienan *et al.* 2002).

1.4 AM host specificity

The vast majority of vascular plants support AM fungal symbioses, however, non-hosts include most plants in the families Brassicaceae, Chenopodiaceae, Cyperaceae, Juncaceae, and Caryophyllaceae (Gerdemann 1968). Due to the biotrophic nature of AM fungi, plants used in rotation crop systems within these families may detrimentally affect the persistence of the AM inoculum (indigenous or exogenous) and the ability of the symbiosis to develop in the field. Therefore, the use of rotation crops could maintain or multiply AM species introduced through inoculation and increase biodiversity.

Nautral systems may better enable AM symbiotic relationships due to the increased number of plant species (Eom *et al.* 2000), and fewer selection pressures from cultural, fertilization, and pest management practices occurring in agricultural

systems (Johnson *et al.* 1992). AM species exhibit some host plant specificity in undisturbed ecosystems such as tallgrass prairie (Eom *et al.* 2000). This specificity may be due to the increased diversity in AM species present (Chanway *et al.* 1991).

In agricultural systems, monocropping can have deleterious effects on AM fungal development (Strzemska 1975), and the effects can be deepened when non-host crops were used in intercropping systems (Baltruschat and Dehne 1988). Johnson *et al.* (1992) reported that continuous monocropping of soybean or corn lead to neutral or declining yield over time, but with the implementation of soybean-corn rotations, yields and tissue nutrient content increased. This increase may be due to an influence of other host plants supporting different AM species, leading to AM species diversity. Perennial crops have been shown to decrease fungal diversity on an annual basis (Wacker *et al.* 1989), while annual crops involved in a regular rotation system allow for increased diversity due to the various interaction effects exhibited by every crop, whereby some AM fungal-plant combinations are more beneficial than others (Klironomos 2003). Different sporulation rates of the AM species is host dependent and can lead to AM species diversity within the community (Bever *et al.* 1996). Karasawa *et al.* (2001) observed an increase in corn yield and colonization when sunflower was used as a pre-crop. The increase in yield was attributed to the accelerated colonization of the corn from the enhanced AM soil inoculum. In addition to increasing the soil inocula through the use of rotation crops, Dodd *et al.* (1990) found that both inoculation of AM fungi and the use of rotation crops increased the rate of establishment due to the enhancement of plant yields in the early stages of development.

1.5 Mycorrhizal dependency

Host plants exhibit different levels of repression of AM fungi resulting in a phenomenon known as mycorrhizal dependency (MD) of the host. MD is described as the difference in shoot dry weight between mycorrhizal and non-mycorrhizal plants (Plenchette *et al.* 1983). The concept of MD not only includes the dependency related to crops, but also cultivars, as there can be treat variation in responses to AM colonization among cultivars within a plant species and/or cultivar. Linderman and Davies (2004) found that marigold varieties varied greatly in their response to AM inoculation when inoculated with four different AM fungal isolates. Similar findings have been reported for field crops such as corn, soybean, and oats (Koide *et al.* 1988; Khalil *et al.* 1994).

P concentrations have been shown to influence a crop's dependency on mycorrhizal colonization for dry matter production, with MD decreasing with increasing soil P (Sharma *et al.* 2001). Azcon and Ocampo (1981) advised that crop/cultivars intended for use with AM fungi, should be evaluated for their MD over a range of available P concentrations. Crop and/or cultivar mycorrhizal dependency, therefore, is influenced by multiple effects such as plant genetics, soil P fertility, timing and method of application, and AM fungal inoculant (Plenchette *et al.* 1983; Linderman and Davis 2004).

1.6 AM inocula

1.6.1 Indigenous vs exogenous

The dispersion of AM fungi in soils throughout the world, allows for ready-to-use indigenous inocula. Unfortunately, access to an indigenous AM fungal population does not necessarily elicit enhanced plant growth, as the optimal species may not necessarily be present within that population. In agricultural soils, fertilization has been shown to select for less beneficial species due to the prolific growth of these species and reduced crop reliance on AM fungi in nutrient rich soil (Johnson 1993). In soils of high fertility it may be increasingly important to identify the best-suited plant species and AM fungal species to best achieve improved plant growth. The beneficial effects of AM symbiosis can as a result of MD (Linderman and Davies 2004).

Exogenous AM inocula, consisting of a mixture of AM fungal spores, hyphae, and colonized plant roots, offers the flexibility of pairing the best plant species and AM species combinations to increase plant yield. AM inoculation in the field is achieved through species substitution or population increases of naturally-occurring AM fungal species. In greenhouse or nursery operations, inoculation involves the exposition of young plants to AM species in an effort to enhance their viability and yield potential following transplantation. Subhan *et al.* (1988) have shown transplantation shock to be alleviated through AM colonization of micropropagated plants.

1.6.2 Single species vs multiple species inoculants

The ability of single species inocula to enhance plant production is undisputed. In agricultural soils, where selection pressures are great, there may be a need for multiple species inocula to allow species to better adapt to various environmental conditions, achieve a higher consistency in the plant responses (Koomen *et al.* 1987) and overcome problems of less compatible host-AM fungal interactions. AM species diversity has been related to higher productivity. Hart and Klironomos (2003) have shown that AM fungal species are functionally different and their impacts on a host plant may be complementary. Although the quantity of each AM fungal species present in the soil varies, dictated by the fertility and crop history of the soil, they may collectively function to full potential by enhancing plant growth (Fitter 1985). The inoculum level, soil fertility, MD, etc. may work against the full AM effect for any given crop.

1.7 Soil phosphorus

1.7.1 Soil P properties

Soil P is a relatively stable soil element present in various forms of organic and inorganic (Pi) forms. The stability of soil P is largely due to the insolubility or poor solubility of Pi. In acidic soils, Pi is fixed with Ca and Mg (Bolan 1991). The result of this high reactivity is that the soil Pi fraction is largely unavailable to plants. The amount of available soil P is comprised of organic and inorganic compounds, while the labile P pool consists of rapid exchangeable P (Paul and Clark 1989).

The concentration of P in soil solution is normally low, ranging from 0.1 to 1 $\mu g.g^{-1}$ soil (Bolan 1991), although the plant demand is high. Contributions to P uptake in plants from mass flow constitute only a small portion, 1-13% (Tisdale *et al.* 1993), of total uptake. Soil diffusion and root interception account for most of P uptake by plant roots, and due to the high reactivity of P in soil, P diffusion is a very slow process. The involvement of the plant root elongation and rhizosphere-related P mobilization processes become increasingly important to fulfill the plant P requirements.

1.7.2 Soil phosphorus and soil microbes

Plants and soil microbes are both active in the solubilization of soil P. The mineralization of soil P is due to the release of phosphatases, a group of enzymes catalyzing the hydrolysis of phosphate from organic phosphatases (Tabatabai and Bremner 1971). Once thought to possess no extracellular phosphatase activity (Tarafdar and Marschner 1994), AM fungi have demonstrated the ability to hydrolyze soil organic P and take up the resultant Pi (Koide and Kabir 2000).

AM fungi have the ability to exploit an increased area in the rhizosphere by extending their hyphae beyond the reach of the host plant roots, and protruding into small soil pores unavailable to plant roots with a much larger diameter. AM mycelia maintain low internal Pi concentrations due to polyphosphate (polyP) formation (Forse *et al.* 1989), which can lead to an increase of P uptake as much as six times higher than that of roots (Jakobsen *et al.* 1992a). Most cases of AM fungi

mediated plant P uptake increase happen in soils of low nutrient availability, in particular P.

1.7.3 Phosphorus effects on AM fungi

High soil P concentrations have been shown to inhibit AM colonization of plant roots, growth of ERM, sporulation and plant responses (Abbott and Robson 1991); Bethlenfalvay 1992; Lui *et al.* 2000; Trimble and Knowles 1994; Olsson *et al.* 2002) compared to AM fungi exposed to low concentrations of soil P. Decreasing root colonization results from increasing soil P concentrations (Bolgiano *et al.* 1983) and P fertilization (Koide and Li 199). It has been suggested that this phenomenon is the result of decreased fungal growth (Bethlenfalvay *et al.* 1983; Amijee *et al.* 1993), in a process regulated by high P concentrations within the plant roots (Menge 1978). More recently, Vierheilig *et al.* (2000a) reported that an increase in P level in mycorrhizal roots was not responsible for reduced root colonization, as no evidence of suppression of cyclohexenone derivatives, secondary plant metabolites commonly induced by AM colonization, could be detected in the suppression of root colonization of P supplied plants (Vierheilig *et al.* 2000b). The reduction in ERM length with increasing P concentrations (Olsson *et al.* 2002), and root colonization as a result of localized P sources accessed only by AM fungi (Boddington and Dodd 1998), support the suggestion the two occurrences are related with reduced fungal growth giving rise to reduced root colonization.

In as much as high soil P can affect AM fungal performance, so can low soil P. Low soil P can negatively affect root colonization (Bolan *et al.* 1984; Koide and Li 1990). In the absence of available P, a symbiotic relationship is unnecessary in the case of P uptake. The regulation of this occurrence is not understood, as it is known that AM fungi can sporulate and grow in root culture supplied with media free of P (Olsson *et al.* 2002).

1.7.4 Phosphorus uptake and translocation in AM fungi

Uptake of soil Pi occurs via H+-ATPase transporters localized in the ERM of AM fungi (Harrison and van Buuren 1995; Maldonado-Mendoza *et al.* 2001). Maldonado-Mendoza *et al.* (2001) also observed a regulation of the Pi transporter gene in the ERM in response to environmental Pi conditions. In the ERM, Pi is used to maintain a constant level of Pi in the cytosolic pool to maintain cellular functions (Ezawa *et al.* 2002). NMR studies using ^{31}Phave shown the ERM of *G. intraradices* to store short-chain polyP in vacuoles (Rasmussen *et al.* 2000), which may act as a reserve of PI as observed in some ectomycorrhizal species (Ashford *et al.* 1999). Excess Pi molecules are converted into long-chain polyP (Solaiman *et al.* 1999) and packaged into vacuoles for more efficient transport to the intraradical mycelium (IRM) of the fungus (Klionsky *et al.* 1990).

The vacuoles of AM fungi have previously been described as spherical and not connected, with a suggestion that AM vacuoles accumulate and transport P to the host as polyP (Cox *et al.* 1975). More recently Uetake *et al.* (2002) described the

organelles in living cells as motile tubular vacuoles. As in other fungi, the longitudinal tubular vacuoles may or may not interconnect to the spherical vacuoles depending on location and environmental conditions (Hyde and Ashford 1997; Cole *et al.* 1998), while the distance the tubular transport system can transport polyP is unknown (Allaway and Ashford 2001). The arrangement of longitudinal bundles may allow transport polyP along the lumen of the vacuolar tubes, although cytoplasmic streaming still plays a role in the process. Cytoplasmic streaming was seen in hyphae treated with low P and tubular vacuoles only were seen in this case (Olsson *et al.* 2002). In higher P treatments, more rounded vacuoles linked to tubular ones were found. The exact form of polyP in the vacuoles is unknown.

Once in the IRM, there is a considerably higher proportion of short-chain polyP to total polyP than in the ERM where there is a higher proportion of long-chain polyP to total polyP (Solaiman *et al.* 1999; Ohtomo and Saito 2005). The average chain length in the IRM is 9 Pi residues, while in the ERM it is 13 residues (Viereck *et al.* 2004). For this to occur, either (1) the long-chain polyP is partially hydrolyzed into short chains by polyPhosphatases, before being further hydrolyzed to surrender individual Pi molecules that are transferred to the host (Solaiman *et al.* 1999; Ezawa *et al.* 2002), or (2) long chain polyP acts as a phosphoryl donor for glucose to form glucose-6-phosphate, which must then be metabolized through either glycolysis or the pentose phosphate pathway (PPP).

The transfer of polyP from the fungus to the host is a process still not well understood. It is thought that the exchange occurs at the fungus arbuscular interface with the plasma membrane of the host cortical cell (Giovannetti and Sbrana 1998).

Novel plant Pi transporters localized around the arbuscules (Raush *et al.* 2001; Harrison *et al.* 2002) have been reported, and should allow for the release of Pi from the fungus to the host.

1.7.4.1 Polyphosphatases

Activities of phosphatases, such as alkaline phosphatases (ALP), have been detected around the arbuscules of AM fungi (Gianinazzi *et al.* 1979; Tisserant *et al.* 1993; Ezawa *et al.* 1995), indicating that phosphatases may be involved in Pi transfer from the IRM of AM fungi to host root. Inhibition of the activitiy of ALP with $BeSO_4$, causing a reduction in phosphate efflux and arbuscular phosphate activity, further suggests its involvement (Kojima and Saito 2004). Aono *et al.* (2004) identified ALP genes from the ERM of *G. intraradices* and *Gigaspora margarita.* Although the transcripts from both genes were constitutively expressed in mycorrhizal roots more than spores or the ERM, transcript expression was not influenced by external Pi levels. Previously, ALPs have been shown to have more activity under conditions of P deficiency (Olsson *et al.* 2002; Boddington and Dodd 1999).

Doubts exist as to whether the ALPs are involved in nutrient transfer. Of importance is the affinity for sugar phosphate rather than polyP of a Ba^{2+}-sensitive ALP (Ezawa *et al.* 1999). Larsen *et al.* (1996) found that benomyl inhibited P translocation, but not ALP activity. Also, ALP activity is high during the initial colonization, but not during growth enhancement, raising doubts about its involvement in nutrient transfer (Kojima and Saito 2004).

1.7.4.2 PolyP metabolism via the PPP

PolyP metabolism is a process well understood in prokaryotic systems, but not eukaryotic systems. Polyphosphatases are still required in order to cleave internal and external Pi, with the involvement of two important polyphosphatases: polyphosphate kinase (PPK), and polyphosphate glucokinase (PPGK) (Kornberg *et al.* 1999). PPK synthesizes polyP, using ATP as a phosphoryl donor, while PPGK then transfers a terminal Pi molecule to glucose to obtain glucose-6-phosphate (G6P). G6P is metabolized in glycolysis and the PPP (Fig. 1.1). In the PPP, G6P enters the oxidative portion of the pathway where NAPDH and ribose5-phosphate, a precursor for nucleotide synthesis are produced (Lehninger *et al.* 1993).

Doubts exist about polyP metabolism through this C metabolic pathway in AM fungi due to the fact that PPK and PPGK have not been found in eukaryotes. One report by Capaccio and Callow (1982) reported both enzymes in *G. mosseae*. These results, however, could not be duplicated (Ezawa *et al.* 2002), although a non-specific acid-phosphatase (ACP) with similar activity to PPK has been found in the vacuoles of AM fungi (Ezawa *et al.* 2001).

1.8 C metabolism within AM fungi

In vivo studies have shown that AM fungi obtain most of their carbon from the host root in the form of hexose, with glucose as the major source (Fig. 1.2). Within the IRM hexose is converted to trehalose and glycogen where it can be used for structural and carbon storage compounds (Shachar-Hill *et al.* 1995). Glucose also

enters the PPP as glucose-6-phosphate (G6P) where energy and precursors for nucleotide synthesis are produced and glucose is recycled to maintain hexose levels in the cytoplasm of the IRM. Triacylglycerides (TAG) formed through glycolysis, in the IRM are exported to the ERM (Pfeffer *et al.* 1999) where the stored lipid is used as metabolic precursors for the glyoxylate cycle (Lammers *et al.* 2001) and the TCA cycle, to produce carbohydrates (CHO). CHOs may also be directly transported to the ERM as glycogen (Bago *et al.* 2003). A bi-directional flow between the IRM and ERM exists as glycogen can also be transported back to the IRM from the ERM.

1.9 Linkage between P and C processes in AM fungi

Regulation of P uptake is achieved through C flow from the host roots. Bücking and Shachar-Hill (2005) increased P uptake, allocation, and translocation in response to increased C availability from the host via arbuscular interface. Increased phosphate efflux from excised IRM in the presence of glucose or 2-deoxyglucose has also been found to nearly equal the decreased amount of mycelial polyP (Solaiman and Saito 2001). Nielsen *et al.* (1998) examined respiration for beans inoculated with AM fungi under various P conditions and concluded that in low P soils root C costs are a primary limitation to plant growth.

The relatedness of the two metabolic processes has elicited a new model to be proposed by Bücking and Shachar-Hill (2005) (Fig. 1.3). In this model, cytoplasmic Pi is maintained and also stored in vacuoles to maintain cellular functions and act and a reserve (Ezawa *et al.* 2002; Rasmussen *et al.* 2000). Excess Pi translocated to the IRM continuously flows across the apoplastic interface and eventually is incorporated

into the P sinks of the host. Increased reserves of P within the host would lead to a stimulation of growth, photosynthesis and sucrose release to the apoplastic interface.

The sucrose is hydrolyzed by plant acid invertase to produce hexoses that are metabolized by AM fungi. Hexose is converted to trehalose and glycogen (Shachar-Hill *et al.* 1995), and hexose-phosphates, such as G6P, which may be acted upon by ALPs, or other hexokinases (Solaiman and Saito 2001), thereby releasing individual Pi. The increase in cytoplasmic concentrations of Pi would cause an efflux of Pi from the fungus to the host (Bücking and Heyser 1999). Enhanced hexose supplied to the fungus would increase the supply of TAGs and glycogen to the ERM, enabling an expansion of the ERM with which to exploit P resources.

As previously stated, high concentrations of soil P can inhibit AM colonization of plant roots, sporulation and plant yields. This may be a direct result of reduced C flow from the host to the fungus under increasing P concentrations (Olsson *et al.* 2002). A reduction in C flow from the host may suggest reduced fungal growth, especially in the ERM, leading to reduced colonization rates (Bethlenfalvay *et al.* 1983; Amijee *et al.* 1989).

1.10 General research objectives

Field experiments were developed to examine the effect of AM inoculation on strawberry fruit and plant production in soils of high fertility, in particular P. A study was also conducted on colonized transformed carrot root cultures to determine the effect of environmental P on the C metabolism of AM fungi.

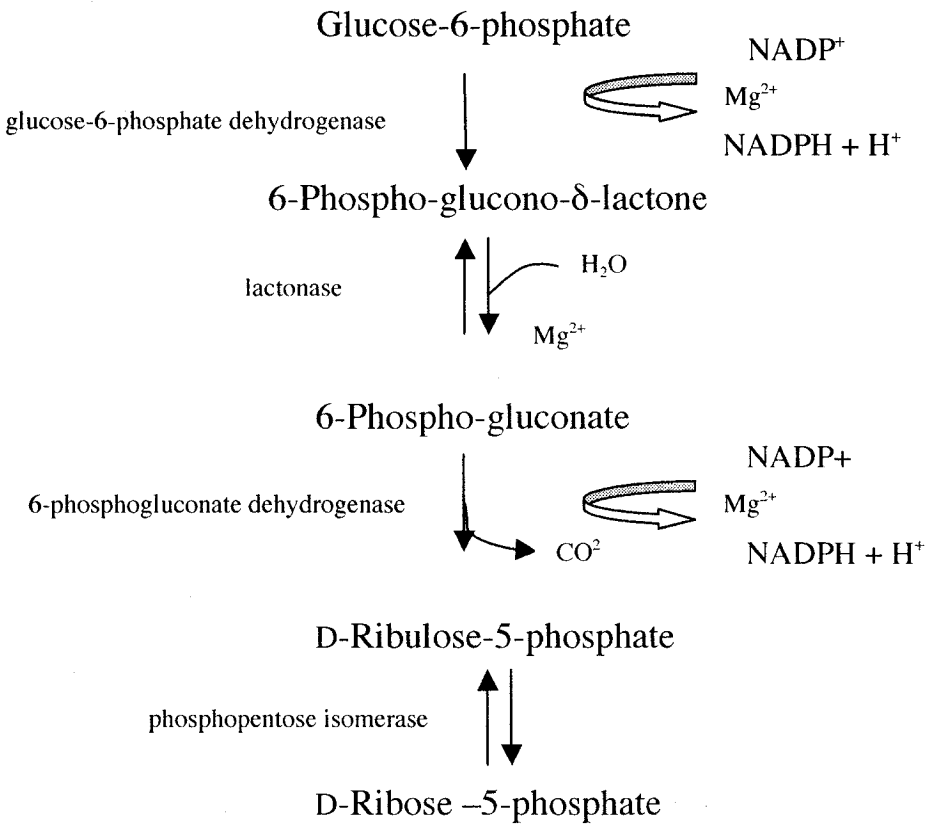


Fig. 1.1 The oxidative reactions of the pentose phosphate pathway, leading to D-ribose-5-phosphate and producing NADPH.

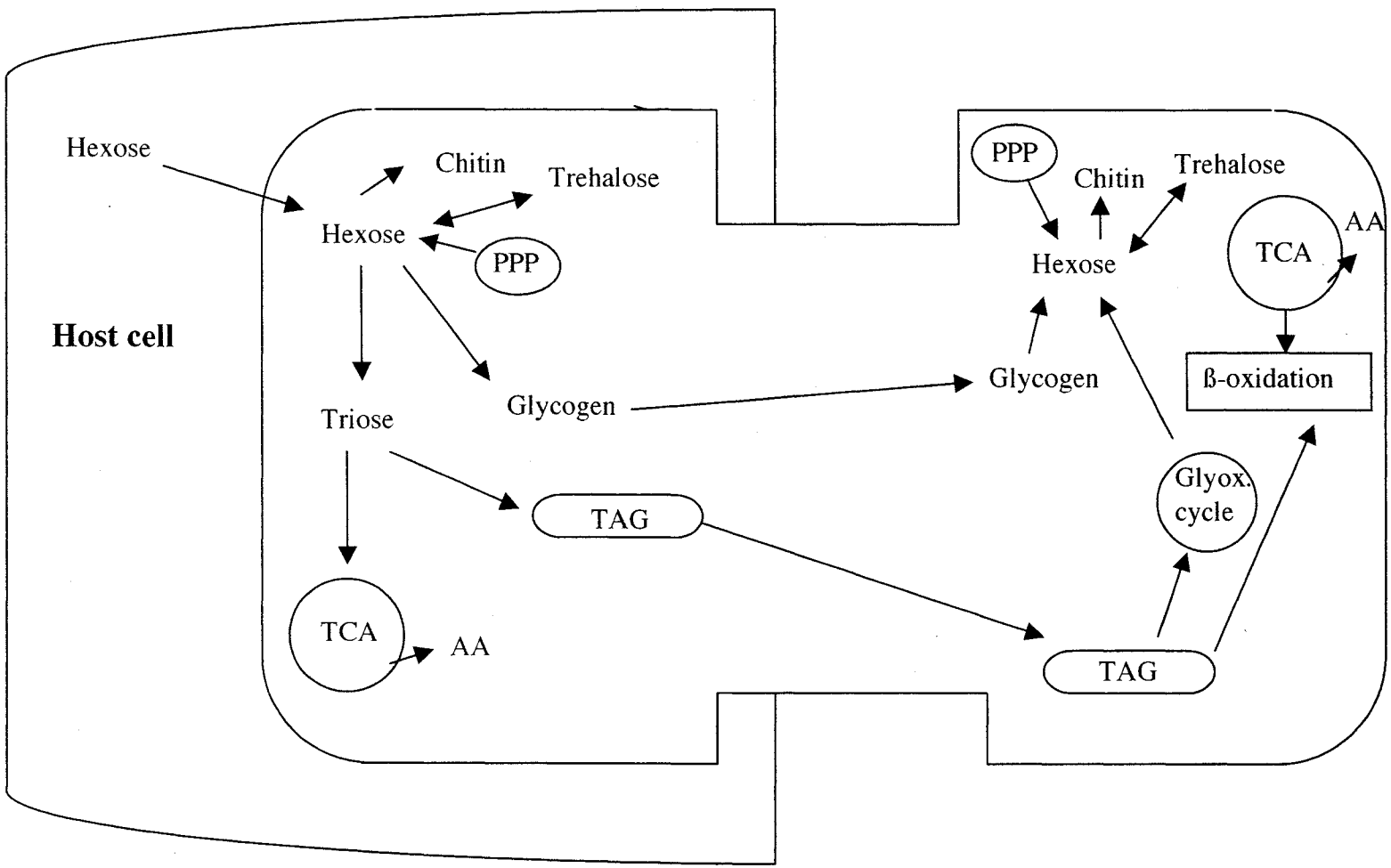

Hexose
Chitin
Trehalose
Hexose
PPP
Host cell
Triose
Glycogen
TAG
TCA
AA
PPP
Chitin
Trehalose
Hexose
TCA
AA
Glycogen
ß-oxidation
Glyox. cycle
TAG
Intraradical mycelium
Extraradical mycelium

Fig. 1.2 Illustration of a model for the C metabolic pathway in the AM fungal symbiosis, adapted from Pfeffer *et al.* (2001). AA, amino acid; PPP, pentose phosphate pathway; TAG triacylglycerides; TCA tricarboxylic acid cycle.

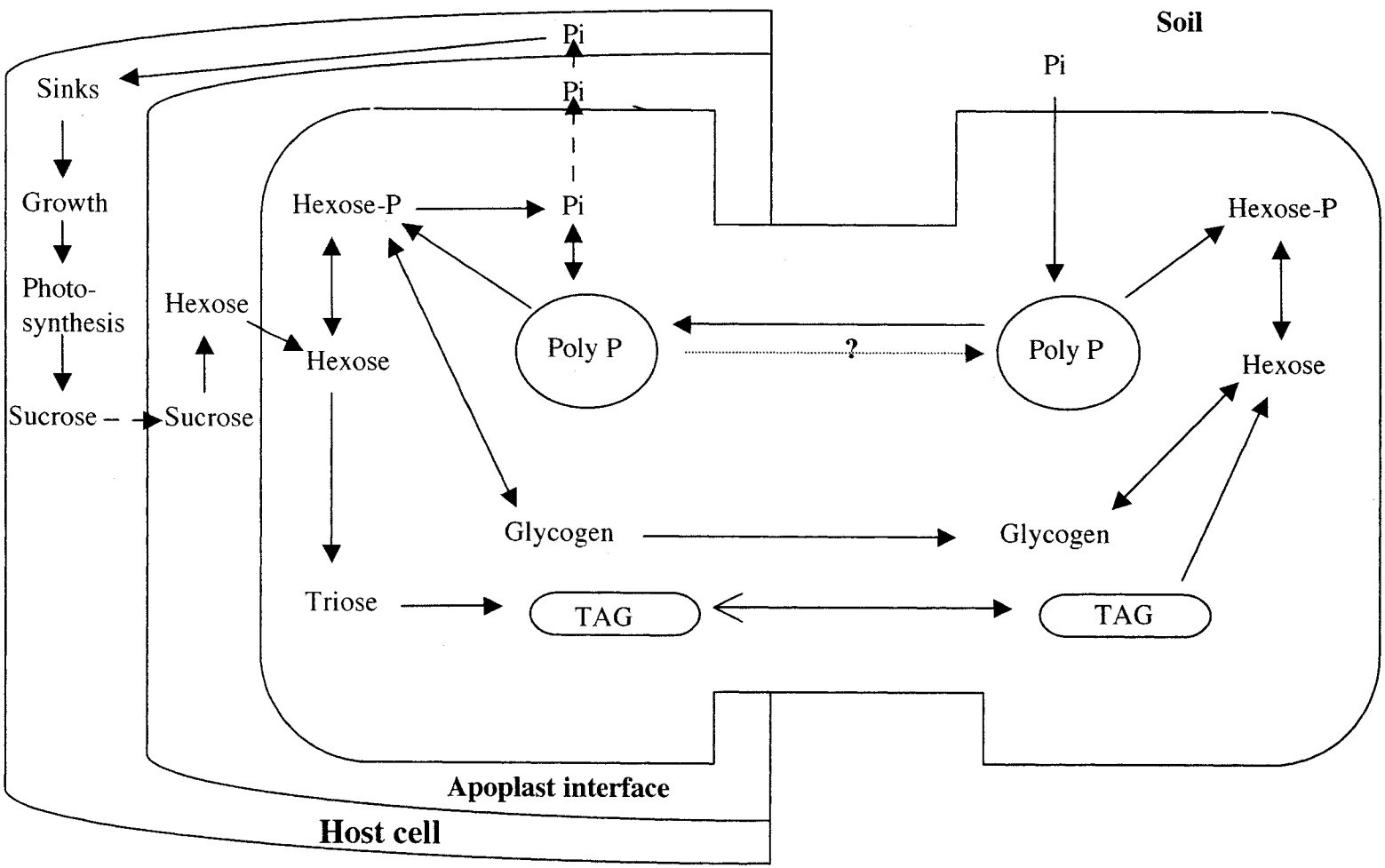

Soil
Pi
Pi
Pi
Sinks
Growth
Photo-
synthesis
Sucrose
Sucrose
Hexose
Hexose-P
Pi
Hexose
Hexose
Poly P
?
Poly P
Hexose-P
Hexose
Glycogen
Glycogen
Triose
TAG
TAG
Apoplast interface
Host cell
Intraradical mycelium
Extraradical mycelium

Fig. 1.3 Illustration of a model for the linkage of the C and P processes in the AM symbiosis, adapted from Bücking and Shachar-Hill (2005). Solid arrows indicate active uptake processes; broken line arrows indicate passive efflux processes. Pi, inorganic phosphate; PolyP polyphosphate; TAG, triacylglycerides.

Connecting Text

The work performed in Chapter 2 was designed to assess the potential for AM fungal inocula use in conventional agriculture production. Specifically, the potential benefit of AM inoculation of elite strawberry plants on plant multiplication in high fertility soils was assessed. Lynda Irene Stewart performed all of the experimental work, accompanying analyses, and writing of the manuscript. Dr. Chantal Hamel, Dr. Richard Hogue, and Peter Moutoglis suggested ways in which to collect field data, and provided critical reading of the manuscript.

Chapter 2. Response of strawberry to inoculation with arbuscular mycorrhizal fungi under very high soil phosphorus conditions

This chapter was adapted from the following:

Stewart LI, Hamel C, Hogue R, and Moutoglis P. 2005. Response of strawberry to inoculation with arbuscular mycorrhizal fungi under very high soil phosphorus conditions. Mycorrhiza DOI 10.1007/s00572-005-0003-z.

2.1 Abstract

A field study was done to assess the potential benefit of AM inoculation of elite strawberry plants on plant multiplication, under typical strawberry nursery conditions and, in particular, high soil P fertility (Mehlich-3 extractible P = 498 mg kg^{-1}). Commercially *in vitro* propagated elite plants of five cultivars ('Chambly', 'Glooscap', 'Joliette', 'Kent' and 'Sweet Charlie') were transplanted in non-inoculated growth substrate or in substrate inoculated with *Glomus intraradices* or with a mixture of species (*G. intraradices*, *G. mosseae* and *G. etunicatum*) at the acclimation stage, and grown for six weeks before transplantation in the field. We found that AM fungi can impact on plant productivity in a soil classified as excessively rich in P. Inoculated mother plants produced about 25% fewer daughter plants than the control in Chambly (P = 0.03), and Glooscap produced about 50% more (P = 0.008) daughter plants when inoculated with *G. intraradices*, while the

productivity of other cultivars was not significantly decreased. Daughter plant shoot mass was not affected by treatments, but their roots had lower, higher or similar mass, depending on the cultivar-inoculum combination. Root mass was unrelated to plant number. The average level of AM colonization of daughter plants produced by non-inoculated mother plants did not exceed 2%, whereas plants produced from inoculated mothers had over 10% of their root length colonized seven weeks after transplantation of mother plants, and approximately 6% after 14 weeks (harvest), suggesting that the AM fungi brought into the field by inoculated mother plants had established and spread up to the daughter plants. The host or non-host nature of the crop species preceding strawberry plant production (barley or buckwheat) had no effect on soil mycorrhizal potential, on mother plant productivity, or on daughter plant mycorrhizal development. Thus, in soil excessively rich in P, inoculation may be the only option for management of the symbiosis.

2.2 Introduction

Conventional agriculture practices for high value crops in North America often include abundant fertilization leading to nutrient accumulation in the soils. In particular, P accumulates in soils with a P fertilization history (Zhang *et al.* 1995). Zhang *et al.* (2004) concluded that large amounts of residual fertilizer P were available in the soil in subsequent years following fertilization due to the slow conversion process of residual fertilizer P to stable P forms. In Quebec, the available P (Mehlich-3 extractible P; Sen Tran and Simard 1993) level of soils associated with strawberry nursery plant production ranged from 63 mg P kg^{-1} to 310 mg P kg^{-1} [i.e., from medium to excessive (CPVQ 2000)] in the spring of 1998 (unpublished), when this study was initiated.

Arbuscular mycorrhizal (AM) root systems are known to support stronger, healthier, higher-yielding plants through increased nutrient acquisition (Bolgiano *et al.* 1983; Johnson et *al.* 1992; Wacker *et al.* 1990; Miller 2000), reduced levels of water stress (Augé 2001), lower disease incidence (Dehne 1982; St-Arnaud *et al.* 1995) or phytohormone production (Allen *et al.* 1980;1982; Shaul-Keinan *et al.* 2002). However, the current perception is that these obligate symbionts play no role in soils where nutrients are highly available (Olsen *et al.* 1999). Improved plant growth in response to AM colonization is mostly achieved in soils when available soil P is limited (Bolgiano *et al.* 1983; Abbott *et al.* 1984; Thompson 1991). AM colonization, sporulation, and plant responses are inhibited by high soil P (Abbott and Robson 1984; Liu *et al.* 2000). The negative effects of soil P on plant response to AM fungi occur even when the condition of high available P is imposed on highly colonized plants (Hamel *et al.* 1996; Dekkers and van der Werff 2000). The level of mycotrophy of the crop explains the extent of the repression (Plenchette and Morel 1996). Fertilization was also found to select for less beneficial AM fungal species due to the prolific growth of these species and reduced crop reliance on AM fungi in nutrient rich soil (Johnson 1993). Therefore, indigenous AM fungal populations in agricultural soils might not be the ones that best enhance plant growth. Thus, in high P soils, the inoculation of mycorrhizae responsive crops with beneficial AM fungal isolates could provide economic benefits. Some AM fungi-plant combinations are more beneficial than others (Klironomos 2003). The selection of beneficial combinations would maximize AM-derived benefits. Thus, it may be profitable to identify the AM inoculant most appropriate for a given cultivar. Biodiversity is related to higher productivity; AM fungal species are functionally different and their impact on a host plant may be complementary (Hart and Klironomos,

2002). A diversity of AM fungal species (indigenous or exogenous) may allow AM fungal populations to better adapt to fluctuating environmental conditions, and achieve a higher consistency in the plant responses (Koomen *et al.* 1987). Thus, a multiple species inoculum could be superior to a single species inoculum.

A most effective AM inoculum would be of little use if the AM fungi it contains does not become established in the field due to the competitive pressure of indigenous AM fungal populations. The introduction of beneficial AM fungal strains through inoculation may be facilitated by reduction of the level of AM inoculum naturally present in a field which may be better adapted to the local condition and out-compete the introduced isolates. The reduction of the level of indigenous AM fungi can be achieved by practices such as fallow (Thompson 1987) or the inclusion of non-host plant species in a crop rotation (Harinikumar and Bagyaraj 1988; Black and Tinker 1979).

Previous studies have shown that AM inoculation of strawberry plants have benefited vegetative growth such as runner formation, number of leaves, leaf area and shoot and root dry matter (Holevas 1966; Daft and Okusanya 1973; Koomen *et al.* 1987; Hrslova *et al.* 1990; Niemi and Vestberg 1992; Vestberg 1992; de Silva *et al.* 1996). Taylor and Harrier (2001) and Khanizadeh *et al.* (1995) found that strawberry vegetative growth response to inoculation depended on cultivar-AM species combinations.

Strawberry nursery plant production is an industry that could be enhanced by AM inoculation techniques. In Canada, elite, or top grade, strawberry plants are produced *in vitro* to insure that plants are free from any deleterious endophytes. The *in vitro* propagated plants are acclimatized in the greenhouse before being sent to specialized nurseries where elite plants will be multiplied vegetatively through above-ground runners, or stolons, from

which new daughter plants are formed in fields that are pathogen-free or fumigated. The daughter plants produced are sold as foundation, or lower grade, plants which are transplanted into strawberry production fields, where they can produce good fruit yields for 1 to 4 years, depending on the cultivar, climate and pest pressure. *In vitro* propagated crops, such as strawberry elite plants, require much less AM inoculum than field crops and can be easily inoculated in the greenhouse, at the acclimatization stage of production, before transplantation into the field for multiplication. Once transplanted, the pre-colonized elite plants would establish a mycorrhizal hyphal network in the soil, which could serve as an inoculum source for the daughter plants.

The objective of this experiment was to assess, under typical commercial production conditions and, in particular, high soil P fertility, the impact of commercially available strawberry cultivars and AM inocula combinations on the productivity of elite strawberry plants and on the mycorrhizal colonization of the daughter plants produced. The effect on the effectiveness of inoculation of preceding host and non-host crop species commonly used in rotation with strawberry production were also tested.

2.3 Materials and Methods

A field experiment was conducted at the Horticultural Research Centre of Macdonald College, McGill University, Ste-Anne-de-Bellevue, Quebec. A site was selected in a Chicot sandy loam soil that was high in nutrient content, in particular P. The experiment had three factors: rotation crop, strawberry cultivar and AM inoculum. Two preceding crops were selected based on their susceptibility to AM colonization: barley is a host plant and buckwheat, a non-host plant. In 1998, the two crops were randomized into

blocks consisting of two 36 m x 6 m plots. Barley and buckwheat were sown in early June 1998 at a seeding rate of approximately 50 kg ha^{-1} and 108 kg ha^{-1}, respectively. Both crops were sown with a row width of 15 cm. Due to the adequate nutrient content of the soil, no additional fertilizer was applied. In the spring of 1999, five strawberry cultivars (Chambly, Glooscap, Joliette, Kent, Sweet Charlie) were pre-inoculated in the greenhouse with one of three AM fungal treatments (control, *Glomus intraradices* Schenck & Smith, or a mixture of *G. intraradices*, *G. mosseae* Gerdemann & Trappe, and *G. etunicatum* Becker & Gerdemann; beneficial isolates selected by Premier Tech Inc.) and transplanted in the field with the factorial combinations (cultivar x inoculum) randomized in sub-plots within the main plots. The strawberry plants were transplanted 0.5 m apart, as prescribed for strawberry plant production, into two 10 m rows 1.0 m apart, which constituted each sub-subplot. Each treatment combination was replicated four times. In the fall of 1998, straw was applied uniformly on the field soil and removed in the spring of 1999. To prevent fruit formation and stimulate runner and daughter plant production, the flower buds were removed from all strawberry plants as they formed, as it is done in commercial nurseries.

2.3.1 Inoculation of strawberry seedlings

Elite class strawberry (*Fragaria* x *ananassa* Duch.) seedlings were obtained from *in vitro* cultures prepared by Phytoclone, Inc. (St-Étienne-des-Grés, Québec). At the acclimation stage, the seedlings were inoculated by transplanting into PRO MIX BX® (PremierTech, Rivière-du-Loup, Quebec), a peat based plant growing mix (10% peat, 90% mycorrhizal spores; pH 5.5-6.5) to which a minimum of one effective AM fungal propagule per gram of growing mix was added. Non-inoculated seedlings were transplanted into

uninoculated PRO MIX BX®. All plantlets were placed in a greenhouse and maintained for two weeks under a white plastic hood providing 45% shading. The plantlets were misted two to four times per day depending on sunlight intensity. Plantlets did not receive fertilizer during this period. Over the two-week period, the hood was gradually removed and the misting was reduced until the plantlets became completely exposed to the greenhouse environment. The temperature in the greenhouse fluctuated between 25°C/18°C for the daytime/nighttime periods. The photoperiod was gradually increased to 16 hours using HPS 400 W (30W/M2) lamps. Following the two weeks under the hood, acclimation to fertilization began with 150 ppm of N in the form of calcium nitrate, potassium nitrate and magnesium nitrate applied twice per week. Fertilizer applications continued for six weeks with formulations of 20-5-30 and 15-0-15 with the application of 40 ppm of N using 4-25-35 during the final week. Following four weeks under greenhouse conditions, the temperature was adjusted to 18°C/15°C daytime to nighttime periods, respectively. The plantlets were irrigated on an as needed basis.

2.3.2 Data collection

Prior to seeding of the rotation crops, a soil analysis was done. Soil available P, K, Ca and Mg was measured using the Mehlich-3 extracting solution (Mehlich 1984). Buckwheat and barley were plowed in as green-manure crops before seed onset. Shoot biomass was measured by sampling the shoots of barley and buckwheat in three 1 m^2 quadrants and drying at 70°C for 24 hours before weighing.

In the spring of 1998 the mycorrhizal potential of the soil was tested by bioassay. The mycorrhizal potential was determined by taking three undisturbed soil cylinders

samples (458 cm^3 of soil) per block. Two one-week-old sorghum plants were transplanted into each cylinder, then placed in the greenhouse and maintained for five weeks. The cylinders were watered as necessary from the bottom to prevent crusting of the soil surface. At the end of the five weeks, the roots were harvested from the cylinders, washed and cut into 1-1.5 cm pieces. The roots were cleared by autoclaving in 10% KOH for 12 minutes and staining with 0.02% acid fuchsin (Brundrett 1994) in lactic acid, glycerol, and water (1:1:1). Percent colonization was determined using the grid-line intersect method, as described by Giovannetti and Mosse (1980).

Root colonization of the strawberry transplants was determined prior to transplanting. Daughter plant colonization was determined at 7 and 14 weeks after planting by randomly sampling five daughter plants per subplot.

The total number of daughter plants was counted in 2 m length rows to determine the average number of daughter plants per mother plant produced. From the 2 m subplot, ten daughter plants were randomly selected for crown measurement, and fresh and dry root and shoot masses measurements.

2.3.3 Statistical analyses

Analysis of variance was done with Network JMP 3.2.6 using the following model:

Y = PC + bloc[PC] + cultivar + PC*cultivar + bloc*cultivar[PC] + inoculum + PC*inoculum + cultivar*inoculum + PC*cultivar*inoculum,

where PC stands for preceding crop. When the analysis indicated differences among the means, means were compared using contrasts. The Shapiro-Wilk W test for normality was conducted on the data prior to analysis. The distribution was not normal for the variable

daughter shoot dry mass. To meet the requirement of the ANOVA test, a natural log transformation was performed on the data set prior to analysis. The distribution was not normal for the percentage of colonization of daughter plants 7 and 14 weeks after transplantation of the mother plants and no transformation could bring normality. The distribution was normal when the data related to the control inoculum treatment were removed from the colonization at 7 weeks data set. Thus, an ANOVA was conducted on this partial data. The percentage of colonization at 14 weeks could not be analyzed by ANOVA; standard errors were computed.

2.4 Results

2.4.1 Soil analysis

The soil analysis revealed that the soil had previously been over-fertilized. This was expected as the field had a history of repeated compost applications in the years preceding this study. The near neutral pH (7.17) Chicot sandy loam was found to have 498 mg P kg^{-1} and 324 mg K kg^{-1}, 2731 mg Ca kg^{-1}, and 377 mg Mg kg-1. As expected in a high P soil, the indigenous mycorrhizal population had a low mycorrhizal potential of 3.32%.

2.4.2 Impact of treatments on AM colonization

The root colonization rate of the inoculated strawberry mother plants, prior to transplantation, never exceeded 4% and non-inoculated plants had no trace of mycorrhizal structure. The percentage of daughter plant root colonization at 7 weeks following transplantation was affected by a cultivar-inoculum interaction (Table 2.1). While *G.*

intraradices produced higher percentage of root colonization in Glosscap ($P = 0.002$) and Joliette ($P = 0.04$) 7 weeks after transplantation, the two infective inocula produced similar colonization levels in Chambly, Kent and Sweet Charlie (Fig. 2.1a). Less than 2% of AM colonization was found in the roots of control plants at that time. Fourteen weeks after transplantation, AM colonization had declined in all treatments except in the uninoculated controls (Fig. 2.1b). *G. intraradices* produced the highest level of colonization in Chambly, Glosscap and Sweet Charlie roots (Fig. 2.1b). AM colonization levels were similar in Joliette and Kent. The significance of these effects could not be tested by ANOVA as data distribution was not normal and could not be normalized by transformation. Colonization percentages were all below 12%. It is noteworthy that uninoculated plants developed only trace levels of colonization (less than 3%) even after 14 weeks in the field. The preceding crops did not influence AM development (Table 2.1).

2.4.3 Impact of treatments on plant multiplication

Strawberry plant productivity was measured as the number of daughter plants produced per mother plant. There was a cultivar x inoculum interaction for the number of daughter plants produced per mother plant (Fig. 2.2). Inoculation generally reduced the number of daughter plants produced per mother plant, although these reductions were significant only in Chambly ($P = 0.03$). Glooscap, in contrast, produced about 50% more daughter plants when pre-inoculated with *G. intraradices* ($P = 0.004$). Marketable plants are all healthy plants with a crown diameter of 8-13mm (R. Hogue personal communication) and all plants randomly chosen from all treatments produced crowns within this range.

The mean shoot dry mass of daughter plants was not affected by treatments (Table 2.1). A cultivar by inoculum interaction (Table 2.1) revealed that while the root dry biomass of Kent daughter plants was reduced (P = 0.003) by both AM inocula, the mixed inoculum increased root biomass production of Joliette daughter plants (Fig. 2.3). Plant production was not influenced by soil moisture, as all treatments received adequate water through irrigation.

2.4.4 Impact of preceding crop

Barley and buckwheat crops were planted into the field to increase or decrease the size of the indigenous AM population prior to introducing selected AM fungal species through inoculation of the subsequent strawberry crop. The spring following these preceding crops, no difference in the soil mycorrhizal potential was found. After growing sorghum plants for five weeks in undisturbed soil cores taken from the barley and buckwheat plots, only 3.8 % and 3.9 % of AM colonization was found in the roots of the trap plants. Shoot dry mass produced by the two crops in 1998 was also not significantly different (barley, 1.88 ton ha^{-1}, buckwheat 1.39 ton ha^{-1}). The only impact of preceding crop was seen on Joliette daughter plant root mass, which was higher (P = 0.00003) after barley than after buckwheat.

2.5 Discussion

The % colonization rates of the strawberry roots of <12% were much lower than those reported in previous greenhouse studies where lower concentrations of P were used. For example, Chavez and Ferrera-Cerrato (1990) obtained colonization levels of 25-75% in

inoculated strawberry plants. Kiernan *et al.* (1984) observed colonization rates of at least 26.6% when strawberry plants were inoculated with *G. mosseae*.

In spite of the low colonization levels obtained in this study, it appears that a functional mycorrhizal network was present and operating. This is evident from the higher % root colonization rates of the inoculated versus uninoculated AM treatments at 14 weeks. The AM fungi carried in colonized mother plants appear to have spread in the soil to reach the daughter plants and increase the level of colonization of daughter plants produced by the latter. The decline in % root colonization between 7 and 14 weeks may be due to the fact that daughter plants are produced at greater distance from the point of AM fungi introduction, the mother plants, as the season progresses (Fig. 2.4). The low level of colonization of daughter plants of later cohorts may be reducing the plot mycorrhizal colonization average. Decline in root colonization with time may also be function of the natural biological decline of the fungi after 14 weeks in a highly P fertile soil. High soil P level reduces both intra- and extraradical AM development (Abbott and Robson 1984; Liu *et al.* 2000). The higher daughter plant mycorrhizal colonization rates sometimes seen in *G. intraradices* inoculated plots at 7 and 14 weeks suggests that *G. intraradices* extraradical mycelium tend to spread over longer distance than that produced upon inoculation with the mix of *G. intraradices*, *G. mosseae* and *G. etunicatum*, or to better resist high soil P fertility than other species.

Daughter plants well colonized by effective AM symbionts may survive transplantation better. Colonization of transplants was shown to improve plant survival after transplantation (Carpio *et al.* 2003; Colozzi-Filho *et al.* 1994; Khaliel and Elkhider 1987). Thus, it seems that inoculation of elite plants may result in the production of better quality

foundation plants, upon improvement of plant production and inoculation strategies. The pre-transplant colonization rate of <4% was lower than expected from five weeks of growth in the greenhouse after inoculation. It might be possible to modify nursery inoculation practices to allow for better colonization rates of the nursery plants. For example, higher dose of inoculant could be used. The dose used in this study, although generally adequate for the production of transplants as per Premier Tech Inc. experimental tests, might be sub-optimal in the case of newly rooted *vitro* plants. Normal commercial production practices were used in this study; therefore, fertilization might also be optimized. Increasing the length of plant acclimation time would less likely be economical considering the high cost linked to greenhouse operation. The production of foundation plants well colonized by effective AM fungi should be more successful in soil with lower P levels than the one used in our study.

Previous studies have generally reported a beneficial effect of mycorrhizal inoculation in strawberry vegetative development. Holevas (1966) and Chavez and Ferrero-Cerrato (1990), observed increases in fruit yield as a result of AM inoculation. *G. intraradices* and other single species inocula have been shown to improve stolon production (de Silva *et al.*1996; Khanizadeh *et al.* 1995; Kiernan *et al.* 1984; Chavez and Ferrero-Cerrato 1990). Khanidazeh *et al.* (1995) observed higher stolon production in inoculated strawberry plants and in particular, in Chambly and Kent. Under the high soil P fertility condition of our study, the effect of inoculation on daughter plant production was positive only when Glooscap was inoculated with *G. intraradices*. In this case, however, productivity increased by about 50%. It appears that large economic benefit can result from the inoculation of Glooscap elite plants. The cultivar Chambly, was negatively affected by AM

inoculation. For cultivars under high soil P conditions, the AM fungi may be acting as an energy drain on the plants; the output of reduced C from the plant may exceed the benefit linked to the presence of the fungi (Graham *et al.* 1997).

The effect of multiple AM fungal species colonizing a host plant is not necessarily additive. On the contrary, Davies *et al.* (2000) report that a mix of AM fungal species reduced growth of pepper plants as compared to inoculation with *G. fasciculatum* alone. The mixed inoculant, however, was more efficient at reducing drought stress (Davies *et al.* 2002), emphasizing that the response of a host plant to AM colonization is not only cultivar or isolate specific, but also depends on the environment. Under conditions typical of commercial strawberry plant production, we found that the single species inocula, *G. intraradices*, was not different in its effect on daughter plant production from that of the mixed *Glomus* species inocula, although only *G. intraradices* increased plant productivity significantly in Glooscap.

The lack of AM inoculation effect on shoot dry weight is a contrary finding to previous greenhouse studies, using lower P concentrations in the potting media, where AM inoculation increased shoot dry weight of strawberry daughter plants (Kiernan *et al.* 1984; Khanizadeh *et al.* 1995), and cultivar-AM interaction effects influenced shoot dry weights (Chavez and Ferrera-Cerrato 1990). Khanizadeh *et al.* (1995) reported that AM inoculation also had an effect at nutrient solution concentrations up to 1000 uM P. The root dry weight being influenced by an inoculation-cultivar interaction is, however, consistent with the findings of Chavez and Ferrera-Cerrato (1990). Strawberry plants with high root dry mass may survive transplantation better. In this regard, it might be beneficial to inoculate elite plants of the cultivar Joliette, as it produced daughter plants with larger root mass, thus,

plant of better quality. In contrast, inoculation of the cultivar Kent resulted in daughter plants with lighter root mass.

Thus, it appears that if AM inoculation technology was to be implemented in strawberry plant multiplication nurseries with high P fertility soils, it should be used only with responsive cultivars. Furthermore, care would have to be taken to use the inoculum appropriate to a given cultivars. In this study, only Glooscap and Joliette benefited from inoculation. Kent always responded negatively to inoculation and inoculated Chambly produced less daughter plants, whereas Sweet Charlie showed no positive or negative growth response. However, inoculation in Chambly and Sweet Charlie led to the production of daughter plants with the largest AM colonization levels.

The only impact of preceding crop was a higher root mass production in Joliette daughter plants after barley than after buckwheat. This effect was unrelated to AM development. The lack of response in AM development to barley and buckwheat as host and non-host preceding crops, is in contrast to previous studies where host plants enhanced AM fungal development thereby increasing plant productivity of the subsequent crop (Dodd *et al.* 1990; Thompson 1991; Gavito and Miller 1998; Karasawa *et al.* 2001) and non-host depleted the soil of indigenous AM species (Harinikumar and Bagyaraj 1988; and Black and Tinker 1979). Management of AM fungal species through selective use of AM host preceding crops leading to net benefits in the subsequent crops has been effective in low-input agriculture (Panja and Chaudhuri 2004). The lack of a preceding crop effect in this study suggests that high P soils, with a low indigenous AM background, requires the introduction of AM fungi through inoculation to achieve AM development and benefits in a given crop.

2.6 Conclusion

We found that AM inoculation may trigger a response on strawberry plant productivity even in excessively P rich soil, a condition not uncommon in Quebec nursery fields. This response was cultivar x AM inoculum specific, and could be large and positive or negative. Thus, care should be taken to select the proper inoculum on responsive cultivars to improve rather than reduce the profitability of nurseries with high P fertility soils. The single species *G. intraradices* inoculant was more effective at increasing daughter plant production than a species mix, in this study. The extremely low infective capabilities of the indigenous AM fungal population studied suggests that inoculation should be preferred over AM population management through crop rotation, in excessively high P soils.

Table 2.1 P values taken from ANOVAs conducted on root and shoot dry mass, number of daughter plants per mother plant, and AM colonization of daughter plants at 7 and 14 weeks from transplantation. These mother plants were different *in vitro* propagated elite strawberry cultivars inoculated or not with a single-species or a three-species mix inoculum, at the acclimation stage in the greenhouse.

Source	**df**	**Root mass**	**Shoot mass**	**Daughter plants produced**	**AM root colonization**	
					7 weeks*	**14 weeks****
Preceding Crop (PC)	1	ns	ns	ns	ns	-
Cultivar	4	0.01	ns	0.008	<0.0001	-
Inoculum	2	ns	ns	ns	<0.0001	-
Cultivar x Inoculum	8	0.05	ns	0.03	0.0003	-
PC x Cultivar	4	0.002	ns	ns	ns	-
PC x Inocula	2	ns	ns	ns	ns	-
PC x Cultivar x Inoculum	8	ns	ns	ns	ns	-

* Data related to control plots were excluded from the ANOVA to meet the normality requirement of the test.

** Normality could not be obtained for this data, thus, was not analyzed by ANOVA.

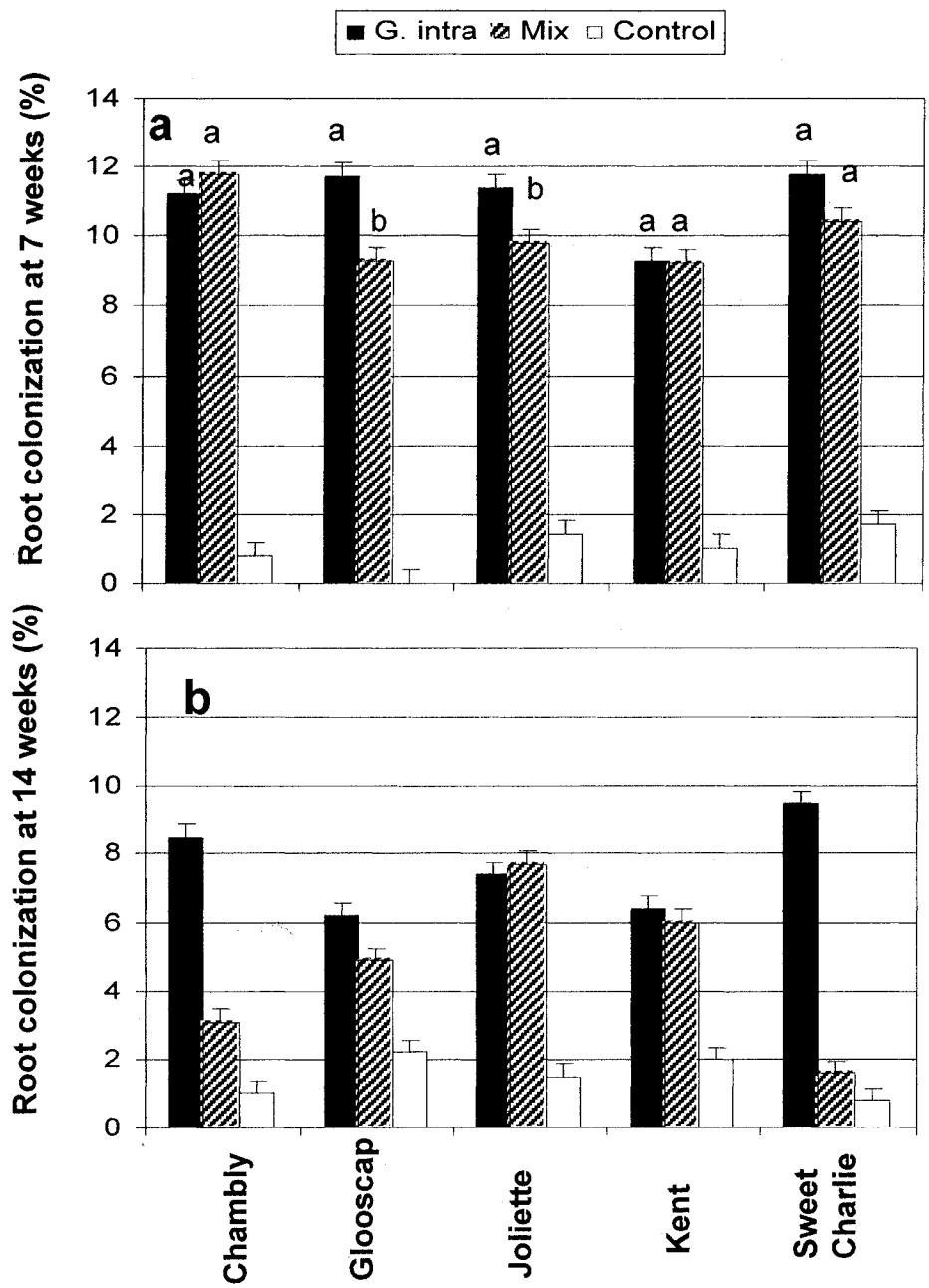

G. intra
Mix
Control
a
b
Root colonization at 7 weeks (%)
Root colonization at 14 weeks (%)
0
2
4
6
8
10
12
14
a
a
a
a
a
a
a
a
b
b
a
a
Chambly
Glooscap
Joliette
Kent
Sweet Charlie

Fig. 2.1 Daughter plant root colonization rates of five strawberry cultivars inoculated with *G. intraradices* (G. intra), a mix of *G. intraradices*, *G. mosseae*, and *G. etunicatum*, or uninoculated a) 7 weeks and b) 14 weeks after transplantation of their mother plants in the field. Note that the 7-week data related to control plot was excluded from the ANOVA to meet the normality requirement of the test; the 14-week data could not be normalized and consequently was not analyzed by ANOVA. Means (n=4) ± standard error followed by similar letters are not significantly different within a cultivar, according to contrasts (P=0.05).

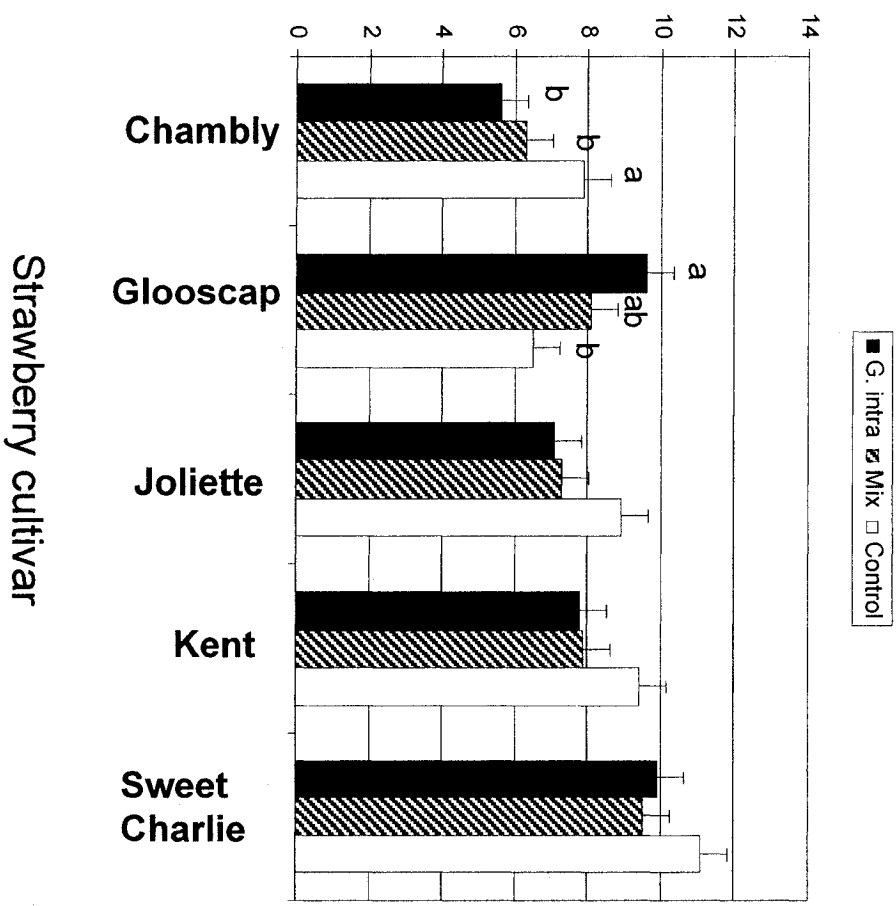

Daughter plants per mother plant
at 14 weeks
Strawberry cultivar
Chambly
Glooscap
Joliette
Kent
Sweet
Charlie
0
2
4
6
8
10
12
14
b
b
a
a
ab
b
G. intra
Mix
Control

Fig. 2.2 Number of strawberry daughter plants produced per mother plant for five strawberry cultivars inoculated with *G. intraradices* (G. intra), a mix of *G. intraradices*, *G. mosseae*, and *G. etunicatum*, or uninoculated. Means (n=4) ± standard error followed by similar letters are not significantly different within a cultivar, according to contrasts (P=0.05).

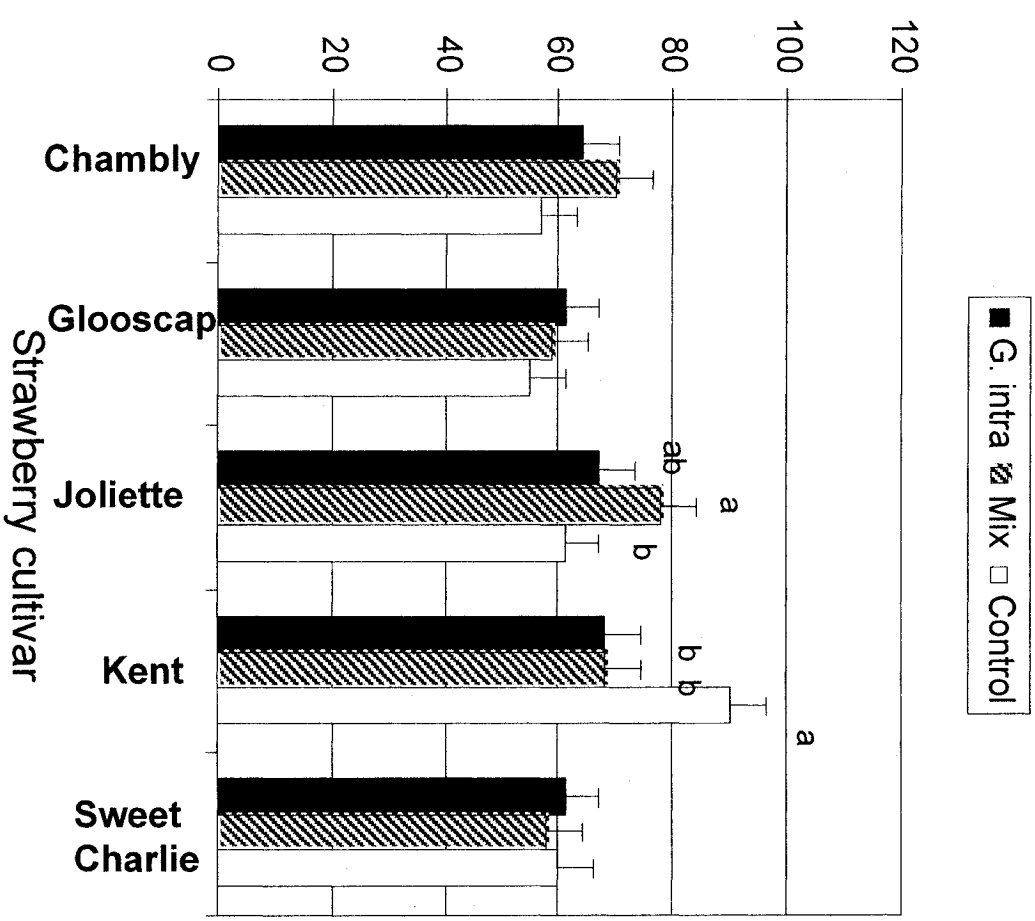

Root biomass (g plant-1) at 14 weeks
0
20
40
60
80
100
120
Chambly
Glooscap
Joliette
Kent
Sweet Charlie
Strawberry cultivar
ab
a
b
b b
a
G. intra Mix Control

Fig. 2.3 Root dry weight of strawberry daughter plant for five cultivars. Means (n=4) ± standard error followed by similar letters are not significantly different within a cultivar, according to contrasts (P=0.05).

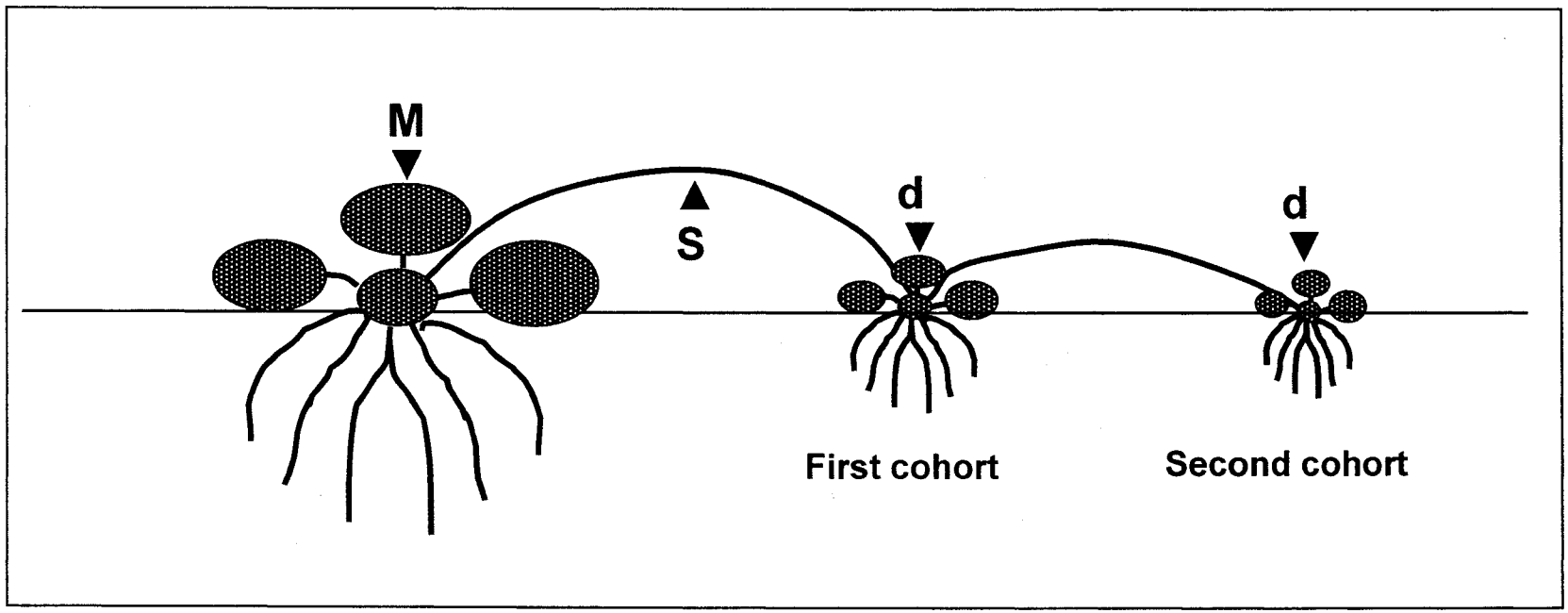

M
S
d
d
First cohort
Second cohort

Fig. 2.4 Diagramatic representation of strawberry vegetative reproduction. M, mother plant; d, daughter plant; S, stolon.

Connecting Text

The work conducted in Chapter 3 is an extension of the work presented in Chapter 2. In this chapter the potential benefit of AM inoculation of elite strawberry plants on fruit yield under conditions typical of commercial fruit production and high soil fertility. Elite strawberry plants were field inoculated following transplantation.

Chapter 3. High soil phosphorus does not inhibit the response to arbuscular mycorrhizal inoculation of field grown strawberry plants

3.1 Abstract

A field study was done to assess the potential benefit of AM inoculation to elite strawberry plants on fruit yield under conditions typical of commercial fruit production and high soil P fertility. Three commercially grown strawberry cultivars ('Glooscap', 'Kent', 'Joliette'), propagated by in vitro, were transplanted into a field comprised of a sandy loam Chicot soil. Approximately four weeks after transplantation, all strawberry mother plants were field inoculated with a noninoculated growth substrate, or a substrate inoculated with either *Glomus intraradices* or *G. mosseae*. In a soil with excessive P levels, AM fungi impacted strawberry fruit yield. During the first year harvest, all inoculated cultivars produced a higher fruit yield than the noninoculated cultivars. Kent was the cultivar that benefited the most from AM inoculation with a 35% increase between noninoculated plants and plants inoculated with *G. intraradices*. The second harvest year was unaffected by AM inoculation although there was an approximate 42% increase in total fruit yield from the previous year. The rate of root colonization remained low throughout the study (<16%), and could not explain the differences in fruit yield during the first harvest year, or the increase in fruit yield of 42% between the first and second harvests.

3.2 Introduction

The ability of arbuscular mycorrhizal (AM) fungi to transport nutrients, such as P, Cu, and Zn, to plant roots, thus enhancing plant yields (Bolgiano *et al.* 1983; Johnson *et al.* 1992; Wacker *et al.* 1990; Miller 2000), has led to the concept of their use as a bio-fertilizer. The hyphal network formed by AM fungi can act as an extension of the plant root system reaching previously unexposed nutrients in nutrient-deficient soils. In sharp contrast to the optimal soil fertility for AM-host symbiosis, is the fertility of North American soils, which are often over-fertilized. Phosphorus is known to accumulate in soils with a P fertilization history (Zhang *et al.* 1995). Zhang *et al.* (2004) concluded that large amounts of residual P fertilizer were available in the soil in the years following fertilization due to the slow conversion process of residual fertilizer P to more stable forms as soil P saturation increases.

While the need exists for more efficient use of soil available P, the excessive soil P levels may impede the use of AM fungi as bio-fertilizers. It has been reported that most plant growth response to AM fungal colonization is enhanced in soils with limited P (Bolgiano *et al.* 1983; Abbott *et al.* 1984; Thompson *et al.* 1991), and AM colonization, sporulation, and plants responses are inhibited by high soil P (Abbott and Robson 1984; Liu *et al.* 2000). The negative effects of soil P on plant response to AM fungi occur even when the condition of high available P is imposed on well colonized plants (Hamel *et al.* 1996; Dekkers and van der Werff 2000), and the extent of repression can only be explained by the mycorrhizal dependency of the

crop (Hamel *et al.* 1991; Plenchette *et al.* 1983). Mycorrhizal dependency alone cannot predict whether a crop will benefit from AM colonization. There can also be great variation in plant response to AM colonization among cultivars within a plant species. Linderman and Davis (2004) found that Marigold varieties varied greatly in their response to AM inoculation when inoculated with four different AM fungal isolates. Similar findings have also been reported for field crops such as barley, corn, soybean, and oats (Koide *et al.* 1988; Baon *et al.* 1993; Khalil *et al.* 1994; Lui *et al.* 2000). Crop and/or cultivar mycorrhizal dependency is, therefore, influenced by multiple effects such as plant genetics, soil P fertility, timing and method of inoculation, and AM fungal inoculant (Plenchette *et al.* 1983; Linderman and Davis 2004).

Abundantly fertilized soils have been reported to select for less beneficial AM species due to the prolific growth of those species and less crop reliance on AM fungi in nutrient rich soil (Johnson 1993). In high P soils, the inoculation of mycorrhizal dependant crops with performant AM fungal isolates could provide economic benefits by identifying the AM inoculant most appropriate for a given cultivar. Strawberry fruit production is a high value industry that could potentially benefit from identifying cultivars and AM inoculants best suited for high soil P conditions.

In Canada, strawberry seedlings are typically transplanted in fruit production fields where they remain for up to four years, with 2-3 years of fruit harvest. This perennial crop may benefit from AM inoculation through an increase in strawberry fruit yield due to an increase of P uptake, or one of the many other benefits

associated with AM symbiosis, such as reduced levels of water stress (Augé 2001), lower disease incidence (Dehne 1982; St-Arnaud *et al.* 1995) or phytohormone production (Allen *et al.* 1980;1982; Shaul-Keinan *et al.* 2002). Dunne and Fitter (1989) determined that P demand of strawberry plants in the vegetative stages of growth is low, but increases later in the season becoming important closer to the flowering phase where the plants' demand exceed its root capacity. Sharma and Adholeya (2004) have shown strawberry fruit yield and unit fruit mass to increase due to AM inoculation and P fertilization up to 150 $kg \cdot ha^{-1}$, but to decrease with higher P fertilizer rates. Therefore, higher P use efficiency of AM inoculated strawberry plants is possible and may lead to reduced fertilizer needs in North America.

The objective of this experiment was to assess, under high soil P fertility conditions typical to those present in commercial strawberry fruit production the potential of AM inoculation of strawberry, a mycorrhizal dependant crop. An experiment was designed to test the following hypotheses: (1) the yield of field-grown strawberry will be increased by AM inoculation even with high soil available P, (2) the AM fungal species that best increases fruit yield will vary with strawberry cultivar.

3.3 Materials and Methods

A field experiment was conducted at the Horticultural Research Centre of Macdonald College, McGill University, Ste-Anne-de-Bellevue, Quebec. A site was selected in a Chicot sandy loam soil high in nutrient content, in particular P. P

availability above the threshold of 400 kg P ha^{-1} is considered excessive for strawberry production (CPVQ 2000). The experiment was arranged in a split-plot design, with two factors: strawberry cultivar and AM inoculum. Three strawberry cultivars (Glooscap, Joliette, Kent) were randomized into the main plots consisting of two 10 m rows with the plants transplanted 0.5 m apart. The strawberry cultivars were inoculated in the field with three AM fungi treatments (control, *G. intraradices* Schenck & Smith, and *G. mosseae* Gerdemann & Trappe) and were randomized within the main plots. The strawberry plants were transplanted into two 10.8 m rows 0.50 m apart. A distance of 1.2 m was left between rows, with an empty border row between treatments to avoid cross-contamination of inocula. The split plot design was replicated four times. Due to the high nutrient content of the field, no addition fertilizer was applied. The field was watered as needed via an irrigation system. Approximately every ten days the plots were weeded. Flower buds were removed during the plantation year to prevent fruit formation and stimulated stolon and daughter plant production. Straw was applied to the plants in the fall of each year to prevent frost damage, and was removed in the spring when the strawberry plants began to produce new growth.

3.3.1 Inoculation of strawberry plants

Elite class strawberry, (*Fragaria* x *ananassa* Duch.) seedlings were obtained from *in vitro* cultures prepared by Phytoclone, Inc. (St-Étienne-des-Grés, Québec). All plantlets were acclimatized in a greenhouse and maintained for two weeks under a white plastic hood providing 45% shading. The plantlets were misted two to four

times per day depending on sunlight intensity. Plantlets did not receive fertilizer during this period. Over the two week period, the hood was gradually removed and the misting was reduced until the plantlets became completely exposed to the greenhouse environment. The temperature in the greenhouse fluctuated between 25°C/18°C for the daytime/nighttime periods. The photoperiod was gradually increased to 16 hours using HPS 400 W (30W/M2) lamps. Following the two weeks under the hood, acclimation to fertilzation began with 150 ppm of N in the form of calcuim nitrate, potassium nitrate and magnesium nitrate applied twice per week. Fertilizer applications continued for six weeks with formulations of 20-5-30 and 15-0-15 with the application of 40 ppm of N using 4-25-35 during the final weeks. Following four weeks under greenhouse conditions, the temperature was adjusted to 18oC/15oC for the daytime/nighttime periods. The plants were irrigated on an as needed basis. The noninoculated seedlings were transplanted into the field in May 1998. On 22 June 1998, mother plants were inoculated with PRO MIX BX® (PremierTech, Rivière-du-Loup, Quebec) a peat based plant growing mix (10% peat, 90% mycorrhizal spores; pH 5.5-6.5) to which one effective AM fungal propagule per gram of growing mix was added. Non-inoculated mother plants were treated with noninoculated PRO MIX BX®. The inoculum was applied to the soil through trenches dug on either side of all mother plants to a depth of 8 cm. Inoculum was added to the trench until it was 1-2 cm thick to form 15 cm bands, with each mother plant receiving approximately 185 ml of inoculum, and then covered with soil.

Daughter plants having formed roots were inoculated on two dates (July and August 1998) to ensure a well-established mycorrhizal network in the inoculated

plots. To inoculate a daughter plant, a small hole was made in the soil within 20 cm on either side of the mother plant. Approximately 100 ml of inoculum was added to the hole, the daughter plant placed in the hole and then covered with soil. The inoculum was equally divided among the subplots to allow the maximum number of daughter plants per subplot to be inoculated.

3.3.2 Data collection

In the spring of each planting season a complete soil analysis was done. Organic matter was measured using the method of Black (1965), and available P, and K using the Mehlich-3 extracting solution (Mehlich 1984).

Mycorrhizal potential of the soil was tested in the spring of the planting year. The mycorrhizal potential was determined by taking three soil cylinder samples (458 cm^3 of soil) per block. Two one-week-old sorghum plants were transplanted into each cylinder, and the cylinders placed in the greenhouse and maintained for five weeks. The cylinders were watered as necessary from the bottom to prevent crusting of the soil surface. At the end of the five weeks, the roots were harvested from the cylinders, washed and cut into 1-1.5cm pieces. The roots were cleared by autoclaving in 10% KOH for 12 minutes and staining with 0.02% acid fuchsin (Brundett 1994) in lactic acid, glycerol, and water (1:1:1). Percent colonization was determined using the grid-line intersect method, as described by Giovannetti and Mosse (1980).

Root colonization was determined at 5 and 13 weeks following transplantation by randomly sampling five daughter plants per plot. During the

second and third year (fruit harvest years) daughter plants were sampled at 4, 8 and 12 week following the removal of straw in the spring.

During the fruit harvest years, the dates of bud, flower and fruit formation were recorded for each subplot when approximately 50% of the plants contained the reproductive structure. This enabled us to determine if differences in plant development were specific to cultivar or AM species inoculation. Every 3-4 days all ripe strawberries were removed, in both harvest years. Total fruit yield and average berry mass were measured for all plots.

3.3.3 Statistical analysis

The Shapiro-Wilk W test for normality was conducted on the data prior to analysis. Square root transformations were conducted on percentage foliar nutrient data and log transformations were conducted, as needed, on root colonization data. ANOVAs were conducted with SAS using the following model:

Y = block + cultivar + block*cultivar + inoculum + cultivar*inoculum

When the analysis indicated differences among the means ($P < 0.05$), means were separated using Duncan's test.

3.4 Results

The soil analysis revealed that the soil had previously been overfertilized (Table 3.1). The results of the soil analysis were expected as the field had a history of repeated compost applications in the years preceding this study.

3.4.1 Fruit yield

Cultivar X inoculum interactions were shown to affect the first year total strawberry fruit yield (Fig. 3.1). Within each cultivar there was a significant difference in yield between inoculated versus noninoculated plants; inoculated plants had a significantly higher yield. The combination of Kent inoculate with G. intraradices produced the highest yield, of approximately 16 000 $kg \cdot ha^{-1}$ (Fig. 3.1), while yields for the treatment combinations of Kent inoculated with *G. mosseae* and Glooscap inoculated with *G. mosseae* were not significantly different ($P<0.05$). *G. intraradices* was a more effective inoculant for increasing fruit yield than *G. mosseae* for the cultivars Joliette and Kent. The difference in yield between the highest producer and lowest producer (noninoculated Glooscap) was an approximate 45% increase. For the second year harvest, the total strawberry fruit yield was not affected by the Am inoculation, cultivar, or any interaction effect (Table 3.2). Although the overall yield had increased by approximately 42% from the previous year, the yield among all treatment combinations had increased to produce similar amount (Fig. 3.1). The increase in yield between first and second harvest year was not be attributed to increased amount of precipitation, as irrigation was provided during both growing seasons. The increase was most likely the result of the strawberry plants achieving their maximum fruit potential in the second harvest year.

Specific berry mass was not influenced by inoculation (Table 3.2). Glooscap had the highest berry mass while Joliette and Kent had a lower berry mass but were not significantly different (Fig. 3.2). As in the first year, the berry mass in the second harvest year was only affected by cultivar (Table 3.2). The average berry mass had also increased from the first year for all cultivars with Joliette increasing by approximately 63% (Fig. 3.2).

3.4.2 Root colonization

During the plantation year, root colonization was not affected by either cultivar or AM inoculation ($P < 0.05$). At 5 weeks following inoculation, the mean colonization rate for all cultivars and treatments was 1.57% (Fig. 3.3). At 13 weeks following inoculation, the mean colonization rate increased to 14.8%.

As in the plantation year, the root colonization was not influenced by cultivar or AM inoculation at the 4, 8 or 12-week sampling periods in the first harvest year ($P < 0.05$). The mean root colonization, which was measured on mother and daughter plants indiscriminately, increased from 7.53% at 4 weeks to a high of 13.7% at 12 weeks (Fig. 3.3).

Due to a loss of samples in the second harvest year, only one set of samples were analyzed for root colonization. The 12 week samples were again unaffected by the cultivar and AM inoculation, but did reach a three-year high mean of 15.2% root colonization.

3.5 Discussion

All strawberry cultivars responded to AM inoculation in a field with a very high P fertility level. The positive responses of all cultivars to inoculation without further fertilization in the first year of fruit harvest, dispels the general notion that AM fungi have no role in soils high in P fertility (Olsen *et al.* 1999). In an effort to reduce fertilizer inputs, this study also indicates that increased yields may be achieved through AM inoculation alone in high fertility soils.

Previously, Chavez and Ferrera-Cerrato (1990) observed an increase in total fruit yield as a result of cultivar-AM interaction. The excessive P level in our experiment of >400 $kg \cdot ha^{-1}$ significantly exceeds the 150 $kg \cdot ha^{-1}$ P fertilizer limit in which Sharma and Adholya (2004) observed increases in strawberry yield and berry mass in response to AM inoculation. Percent root colonization does not explain the any increase in fruit yield during the first harvest year, as the there was no difference between inoculated and non-inoculated treatments. One suggestion for the lack of differentiation between the inoculated and noninoculated treatments may be that indigenous species in the noninoculated subplots have the same ability to colonize the plant roots as the introduced species, but are less effective in providing benefit to the plants. This latter point is evident by a lower yield from the noninoculated plants than the inoculated plants, in the first harvest year, indicating that some fungal-plant combinations are more beneficial than others (Klironomos 2003). In fact, these results re-enforce the concept of cultivar mycorrhizal dependancy as seen with other crops (Koide *et al.* 1998; Khalil *et al.* 1994; Linderman and Davis 2004). The results of our study do not indicate that either *G. intraradices* or *G. mosseae* is a better colonizer or better symbiotic agent for strawberries.

Percent root colonization of the plant could not explain the increase in fruit yield of 42% between the first and second harvests. Percent root colonization was unaffected by the cultivar or AM colonization. As berry mass was found to be affected by the cultivar in both harvest years, an increase in the number of fruit and plant number could account for the overall increase in total yield of 42% between the first and second harvests. Niemi and Vestberg (1992) did not find any AM

inoculation effects on fruit yield. Their study, however, considered only the harvest of the third year, which corresponds to our second harvest year findings.

The colonization rate of the strawberry roots of approximately 15%, at their highest level, is lower than those reported in previous studies with lover soil P concentrations. Chavez and Ferrera-Cerrato (1990) obtained levels of 25-75% in inoculated strawberry plants while Keirnan *et al.* (1984) observed colonization rates of at least 26.6% when *G. mosseae* was used as an inoculant for strawberry plants. Niemi and Vestberg (1973) only observed colonization rates at <14% in the first year of their study. The low rates may be due to the high P level of the soil. High soil P levels reduce both intra- and extraradical AM development (Abbott and Robson 1984; Liu *et al.* 2000). Sharma *et al.* (2001) found significant decreases in colonization in soil with P above 35 ppm when *Albizzia lebbeck* was inoculated with a mixture of indigenous AM fungi.

The in-field inoculation method used in this study may be replaced by greenhouse inoculation. Inoculation of Elite strawberry plants in the greenhouse at time of acclimatization would reduce the amount of inoculant and labour required with field inoculation. Greenhouse inoculation may also allow greater root colonization in strawberry mother plants, as negative soil effects, such as high soil P, could be avoided. Colonization of transplants was shown to improve plant survival after transplantation (Carpio *et al.* 2003; Colozzi-Filho *et al.* 1994; Khaliel and Elkhider 1987). This method should be explored and optimized to benefit producers.

3.6 Conclusion

In a soil with excessively high available P level without additional fertilizer amendments, AM inoculation can increase the yield strawberry fruit. The increase in fruit yield is not attributed to root colonization level, but was dependant on cultivar-AM fungal inoculant specificity, showing variation in the mycorrhizal dependency among cultivars. *G. intraradices* and *G. mosseae*, as introduced species, appear to be more beneficial to strawberry fruit production in a high P soil than the indigenous AM fungal population.

Table 3.1. Soil available P, K, Ca and Mg, organic matter content (O.M.%), pH and mycorrhizal potential of the Chicot sandy loam used for this experiment (n=4).

Nutrient Concentration (kg/ha)					Soil Variables		
	P	K	Ca	Mg	O.M. (%)	pH	Myc. Potential (%)
1998	552	270	3035	284	3.36	6.99	4.1
1999	494	259	3242	275	4.20	7.20	-
2000	510	210	3170	254	4.45	7.16	-

Table 3.2. Significant P values taken from ANOVAs conducted on total fruit yield and average berry mass of AM inoculated Elite strawberry plants (n=4).

Source	df	Total yield 1999	Berry mass 1999	Total yield 2000	Berry mass 2000
Block					
Cultivar	2	<0.05	<0.05	ns	<0.005
Block*Cultivar	6	ns	ns	ns	ns
Inoculum	2	<0.005	ns	ns	ns
Cultivar*Inoculum	4	<0.05	ns	ns	ns

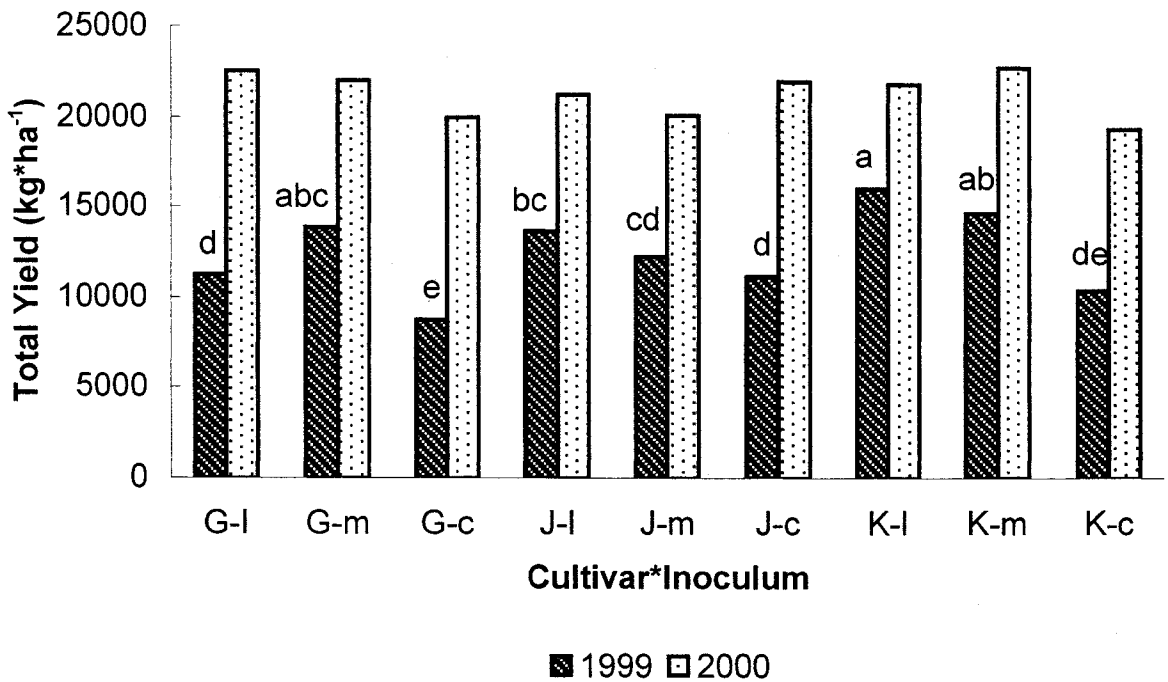

25000
20000
15000
10000
5000
0
Total Yield (kg*ha-1)
d
abc
e
bc
cd
d
a
ab
de
G-l
G-m
G-c
J-l
J-m
J-c
K-l
K-m
K-c
Cultivar*Inoculum
1999
2000

Fig. 3.1 Total strawberry fruit yield for cultivar*AM interactions for two harvest years. G-I, Glooscap-*G. intraradices*; G-m, Glooscap-*G. mosseae*; G-c, Glooscap-noninoculated control; J-I, Joliette- *G. intraradices*; J-m, Joliette-*G. mosseae*; J-c, Joliette-noninoculated control; K-I, Kent- *G. intraradices*; K-m, Kent-*G. mosseae*; K-c, Kent-noninoculated control. Means (n=4) with standard errors followed by similar letters are not significantly different within a cultivar, according to contrasts ($P<0.05$).

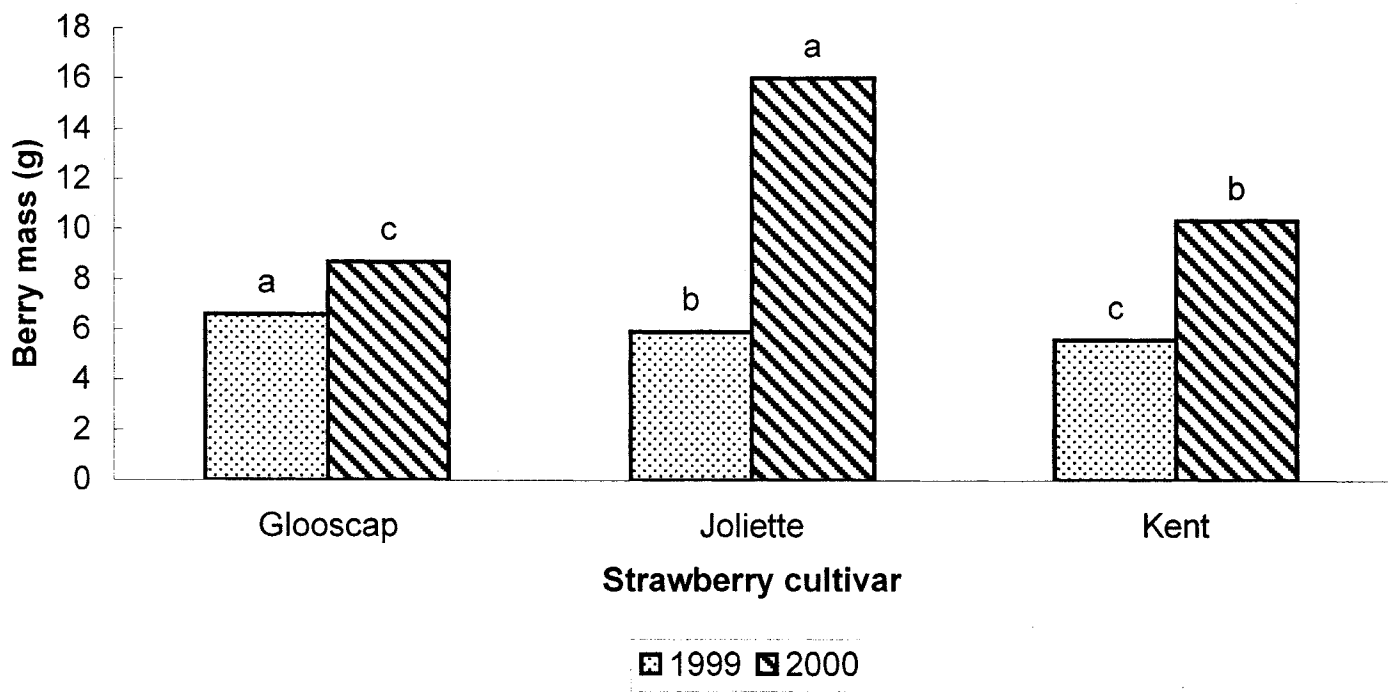

18
16
14
12
10
8
6
4
2
0
Berry mass (g)
a
c
b
a
c
b
Glooscap
Joliette
Kent
Strawberry cultivar
1999
2000

Fig. 3.2 Mean berry mass for three AM inoculated strawberry. Means (n=4) with standard errors followed by similar letters are not significantly different, according to contrasts ($P<0.05$).

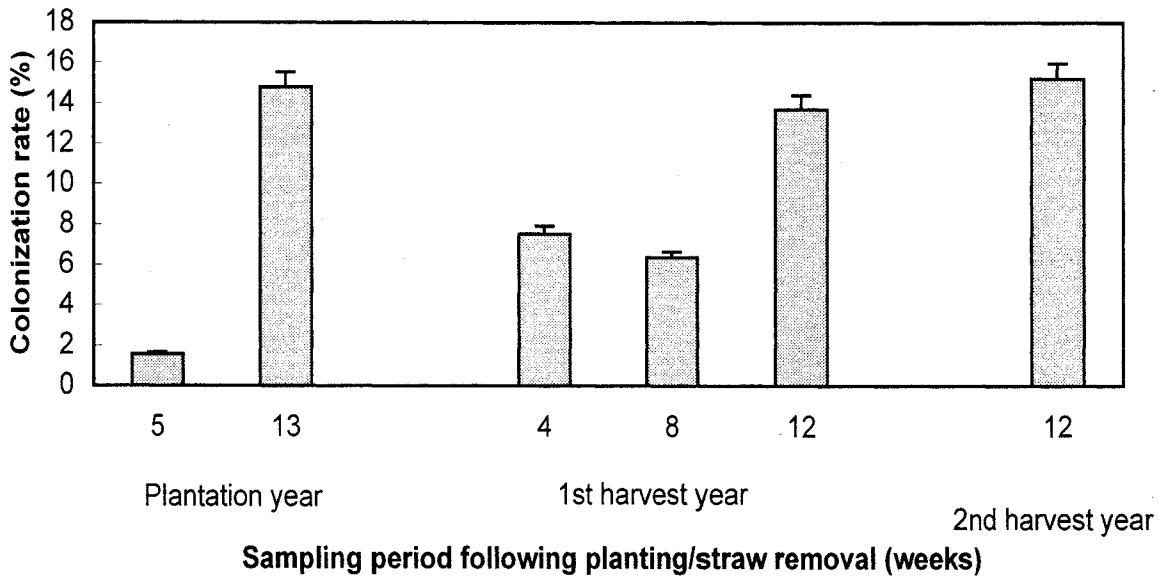

Colonization rate (%)
18
16
14
12
10
8
6
4
2
0
5
13
4
8
12
12
Plantation year
1st harvest year
2nd harvest year
Sampling period following planting/straw removal (weeks)

Fig. 3.3 Mean root colonization rates of three AM inoculated strawberry cultivars. Means (n=4) with standard errors followed by similar letters are not significantly different, according to contrasts ($P<0.05$).

Connecting Text

Upon completion of the field experiments presented in Chapters 2 and 3, it was determined that in high fertility soils inoculation with AM fungi influences strawberry plant and fruit production. To gain more understanding of this plant-fungus relationship under high P conditions, we decided to undertake in vitro experiments involving various levels of P concencentrations and examine their effect on fungal C metabolism. The work performed in Chapter 4 was designed to determine if external P concentrations cause mediated responses in fungal C metabolism during the symbiotic phase of *G. intraradices*. The expression of the *G. intraradices* G6PDH gene was evaluated by real-time quantitative RT-PCR. Lynda Irene Stewart performed the experimental work, accompanying analyses, and writing of the manuscript. Drs. Brian Driscoll and Suha Jabaji-Hare provided critical advice and reading of the manuscript.

Chapter 4. Effects of external phosphate concentration on glucose-6-phosphate dehydrogenase gene expression in the arbuscular mycorrhizal fungus *Glomus intraradices*

4.1 Abstract

Specific primers were developed to amplify a 227-bp segment of the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* gene encoding glucose-6-phosphate dehydrogenase (G6PDH), an enzyme involved in the pentose phosphate pathway. G6PDH gene expression was measured by real-time quantitative reverse transcriptase polymerase chain reaction (real-time QRT-PCR) in response to P concentrations in the growth medium of colonized transformed carrot roots. We wished to investigate the effects of different P concentration treatments on carbon metabolism within the intraradical mycelia (IRM) of *G. intraradices*. The results showed a significant (P=0.017) down-regulation of G6PDH expression in the IRM of *G. intraradices* cultures grown in a high P as compared to low P conditions, but no significant difference in regulation in excessive P concentrations when compared to the low P or high P concentrations. These results indicate that a reduction in the C flow from the host could be occurring as a result of elevated P, and that a decrease in fungal G6PDH gene expression occurs, but not in the short term (less than two hours). Reduced C flow from the host could lead to reduced fungal growth and root colonization, as is observed under high soil P conditions.

4.2 Introduction

Arbuscular mycorrhizal (AM) fungi are obligate symbionts that have been associated with plant roots for over 400 million years (Remy *et al.* 1994). It is currently estimated that over 80% of vascular plants form a symbiotic relationship with AM fungi. Soil nutrients and photosynthetically-reduced carbon are exchanged between the fungus and the host root. Slowly motile nutrients such as P, Cu and Zn, are particularly important in this relationship (Jakobsen *et al.* 1992). Regulation of the nutrient exchange, in particular of phosphate and reduced carbon compounds, between the fungus and the host is still not well understood. *In vivo* studies have shown that AM fungi obtain most of their C from the host root in the form of hexoses, mainly glucose. Within the intraradical mycelium (IRM), which reside inside the host root, hexose is converted to trehalose and glycogen, which can be used for biosynthesis of structural compounds, such as chitin, and carbon storage compounds (Shachar-Hill *et al.* 1995). Triacylglycerides (TAG), synthesized via glycolysis, in the IRM, are exported to the extraradical mycelia (ERM) (Pfeffer *et al.* 1999), where the stored lipids are metabolized via the glyoxylate cycle (Lammers *et al.* 2001) and the tricarboxylic acid (TCA) cycle for the biosynthesis of carbohydrates (CHO). There is evidence that CHOs, in the form of glycogen, are directly transported from the IRM to the ERM (Bago *et al.* 2003).

Inorganic phosphate (Pi) is taken up from the soil by the ERM via active transport, converted into long-chain polyphosphate (polyP) (Solaiman *et al.* 1999), packaged into vacuoles, and then transported to the intraradical mycelia (IRM). Nutrient exchange occurs at the arbuscular interface of the IRM periarbuscular membrane (PAM) with the plasma membranes of host root cortical cells (Giovannetti and Sbrana 1998). In the IRM, the

proportion of short-chain to long-chain polyP is high (Solaiman *et al.* 1999). The short chain polyP may be metabolized either by hydrolysis of exopolyphosphatases, which liberates individual Pi (Solaiman *et al.* 1999), or by transfer of the terminal Pi to glucose, forming glucose-6-phosphate (Kornberg *et al.* 1999). Further metabolism of glucose-6-phosphate would most likely occur through either glycolysis or the pentose phosphate pathway (PPP).

To determine if the fungi utilize either glycolysis or the pentose phosphate pathway for P metabolism when exposed to excess P, it is necessary to examine the expression of enzymes involved in these pathways under different external P conditions. This study focuses on the PPP, in particular glucose-6-phosphate dehydrogenase (G6PDH) which catalyzes the conversion of glucose-6-phosphate + $NADP^+$ to 6-phosphoglucono-δ-lactone + NADPH. Changing external P concentrations may affect the activity of G6PDH and gene expression in the IRM, as these conditions would be expected to affect the amount of glucose-6-phosphate levels due to its production via the PPP. Thus, the objective of this study was to determine if changes in P concentrations in the growth medium alter C metabolism in the AM fungus *Glomus intraradices* during the symbiotic phase. To accomplish this, expression of the gene encoding G6PDH was monitored by real-time quantitative reverse transcriptase polymerase chain reaction (QRT-PCR). QRT-PCR was used to measure the expression of *G. intraradices* genes during the symbiosis as this technique is highly sensitive, requires only small quantities of RNA, and could be used with samples containing both plant and fungal RNA.

4.3 Materials and Methods

4.3.1 Production of fungal material

Pure cultured spores of *G. intraradices* obtained from PremierTech, (Rivière-du-Loup, Quebec) were germinated in liquid M medium without sucrose (St-Arnaud *et al.* 1996) for 10 days at 25°C. The spores were harvested from the liquid medium by centrifugation at 4°C in a Sorvall Superspeed centrifuge at 10 000 rpm for 10 min. The spores were flash-frozen with liquid nitrogen and stored at –80°C until further use.

To multiply existing root cultures of *G. intraradices*, plugs of solid M media containing *Ri*-transformed carrot (*Daucus carota* L.) roots colonized by *G. intraradices* were transferred from 12-week old cultures to Petri dishes containing solid M media (St-Arnaud *et al.* 1996). Uncolonized transformed carrot root cultures were cultured in the same manner. All cultures were maintained for another 12 weeks at 25°C.

4.3.2 Experiment 1 - Growth of AM fungi under various P concentrations

Plugs of solid M medium with colonized and uncolonized transformed carrot roots were transferred to Petri dishes containing approximately 20 ml of solid M media. Three P treatments, in the form of KH_2PO_4, had been applied to the solid media: a low P concentration (LP) of 35 µM P (as 4.8 mg/l KH_2PO_4), high P concentration (HP) of 350 µM P (as 48 mg/l KH_2PO_4), and excessive P concentration (EP) of 5 mM P (as 690 mg/l KH_2PO_4). The LP treatment of 35 µM P is the standard P concentration in M media (St-Arnaud *et al.* 1996), and the EP concentration of 5 mM was previously reported to cause a reduction in the translocation of C to the fungus (Olsson *et al.* 2002). All P treatments were replicated three times. Dishes were sealed with Parafilm, covered with aluminum foil to

exclude light, and maintained for 10 weeks at 25°C in an incubator. Following the ten weeks of growth, RNA was extracted from roots in each of the P treatments so that G6PDH gene expression could be assessed by QRT-PCR.

4.3.3 Experiment 2 – Short-term exposure to additional P

Plugs of solid M medium with colonized and uncolonized transformed carrot roots were transferred to Petri dishes containing approximately 20 ml of solid M media with a P concentration of 35 μM P (as 4.8 mg/l KH_2PO_4). Following 10 weeks growth at 25°C, the root cultures received three additional P treatments: No P, LP (35 μM P (as 4.8 mg/l KH_2PO_4)) and HP (350 μM P (as 48 mg/l KH_2PO_4)) in 2.5 ml of liquid M media pipeted onto the media. All treatments were replicated three times. All root cultures were incubated in the dark for two hours at 25°C. A two-hour incubation period was selected as it was previously shown that maximum accumulation of polyP occurs under three hours (Ezawa *et al.* 2003), and polyP efflux from excised IRM decreased with time over a two hour period when in the presence of glucose (Kojima and Saito 2004). Following the two hour incubation, RNA was extracted from roots in each of the P treatments so that G6PDH gene expression could be assessed by QRT-PCR.

4.3.4 PCR primers

A partial (515-bp) *G. intraradices* G6PDH cDNA sequence and a G6PDH reverse primer G6PDH-rev (5'-CGGCCTCTTGAATCCATATTTAGA-3') were obtained from PJ Lammers (New Mexico State University, Las Cruces, New Mexico). The partial G6PDH cDNA sequence was used to design the G6PDH forward primer G6PDH-LS1 (5'-

TCAGATTTGAAGATTCCCGATGCG-3'). The predicted size of the amplified product is 227-bp. Amplification of two constitutively expressed housekeeping genes (18S rRNA gene and the ß-tubulin gene) specific for *G. intraradices* were used as controls for the real-time QRT-PCR experiments. The primers for the 18S rRNA gene, GIfor (5'-CCTGCTAAATAGCTAGGCCTAAC-3') and GIrev (5'-CTCCGAATCTCAATCCGAAGGC-3') amplified a 362-bp DNA fragment (Filion *et al.* 2003), while primers for ß-tubulin gene, Giβt-for (5'-TACCATGGACTCCGTTCGT-3') and Giβt-rev (5'GACGTGGAAAAGGCACCATA-3') amplified a 575-bp DNA fragment (Delp *et al.* 2003).

4.3.5 DNA sequencing and analysis

DNA sequence reactions (10µl) were done in 96-well plates with BigDye Terminator cycle sequencing reactions (version 2.0 and 3.0) and a 3730XL DNA Analyzer system (PE Applied Biosystems, Mississauga, ON) by the McGill University and Genome Quebec Genome Center. Nucleotide sequences were compiled using Sequencher v. 3.0 (Gene Codes Corporation, Inc., Ann Arbor, MI). Nucleotide and amino acid sequences were compared to the non-redundant (nr) databases using the BLAST algorithms (blastn, blastx, blastp) (Altschul *et al.* 1990). Sequences were aligned using the Clustal W algorithm (Thompson *et al.* 1994), and analyzed using MacVector 7.1 (Oxford Molecular Ltd., Genetics Computer Group, Madison, WI). Included in the multiple alignments are G6PDH sequences (accession numbers in brackets) from *Magnaporthe grisea* (XM_365081), *Emericella nidulans* (X84001), *Neurospora crassa* (XM_331502), *Saccharomyces cerevisiae* (M34709), and *Aspergillus niger* (X87942).

4.3.6 RNA extraction and reverse transcription

The sample preparation of colonized carrot roots included the removal of the ERM. Tissue from colonized and uncolonized carrot roots and separately germinated spores were ground into a fine dry powder using a mortar and pestle. Total RNA was isolated from approximately 100 mg of colonized carrot root using the RNeasy Plant Mini Kit (Qiagen, Mississauga, ON) and was treated using the RNase-Free DNase set (Qiagen), following the manufacturer's instructions. Prior to reverse transcription, all RNA samples were subjected to conventional PCR to ensure no DNA contamination was present in the samples. Spore RNA samples required a second DNase treatment to eliminate all traces of DNA contamination. RNA concentration and quality was determined from the OD_{260}/OD_{280} ratio as measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). To quantify the total extracted RNA the NanoDrop ND-1000 software used the extinction coefficient of 40 μg/ml at an OD_{260} of 1.0. An amount of 0.2 μg of total RNA was reverse transcribed into 20 μl of cDNA using the Omniscript RT Kit (Qiagen) according to the manufacturer's instructions.

4.3.7 cDNA amplification by conventional RT-PCR

To construct standard curves for the target gene and the housekeeping (HK) genes, conventional RT-PCR assays were performed on cDNA templates prepared from total RNA extracted from germinated *G. intraradices* spores using the 18S gene, β-tubulin, and G6PDH primers. cDNA samples were diluted 100-fold with autoclaved Nano-Pure water. Amplification was performed in 50 μl reaction volume containing: 5 μl 10X PCR buffer, 1 μl 10 mM dNTP, 2 μl 25 mM $MgCl_2$, 2.5 μl of 10 μM of each primer, 0.25 μl Taq

polymerase (Invitrogen Life Technologies, Burlington, ON, Canada), 5 µl cDNA, and 31.75 µl PCR water. Amplification conditions for target and HK genes were as follows: 94°C for 10 min, followed by 45 cycles of amplification at 94°C for 150 s, 57°C for 40 s, 72 °C for 60 sec. A final extension step was added at 72 °C for 10 min. RT-PCR products for all genes were purified with QIAquick PCR Purification Kit and quantified using NanoDrop ND-1000 Spectrophotometer. Serial dilutions of the purified RT-PCR products in the range of concentration from 10^1 to 10^8 and over 4 to 5 orders of magnitude were prepared to construct standard curves for QRT-PCR assays. Negative controls containing cDNA with no RT were subjected to the same procedure to ensure no contamination. cDNA from non-colonized transformed carrot roots were included to ensure primer specificity.

4.3.8 Real-time quantitative RT-PCR (QRT-PCR)

QRT-PCR was performed for each of the housekeeping genes, 18S rRNA and β-tubulin, and for G6PDH on cDNA templates extracted from colonized carrot root tissue using the MX3000P Real-Time PCR System (Stratagene, La Jolla, Ca, USA) and SYBR Green PCR master mix (Qiagen). For each gene a set of standard solutions prepared from RT-PCR products of *G. intraradices* spores was included in each run.

Reactions for the β-tubulin, 18S rRNA gene and G6PDH gene were: 10 µl of SYBR Green master mix, 8 µl of PCR water, 0.5 µl of each primer, and 1µl of cDNA for either the samples or standards. The amplification conditions for β-tubulin were: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 60 s, 72°C for 60 s followed by a dissociation incubation for 1 cycle at 95°C for 60 s and 55°C for 30 s. Similar amplification

conditions were used for 18S and G6PDH except the annealing temperature was adjusted to 59°C and 58°C for each gene, respectively. Crossing points (CP) were determined by the threshold cycles (Ct) at a fixed fluorescence of 0.07. The standard curve for each gene was generated by a linear relationship obtained by plotting the Ct numbers versus cDNA initial quantity (ng). Regression analysis was performed to determine the fit of the standard curve to the standard data points. The BestKeeper software (Pfaffl *et al.* 2004) was used to determine the stability of the internal housekeeping genes by comparing the reference gene CP with an index of ten best-suited standards in order to determine if the expression of reference genes are altered under various conditions. The relative expression software tool (REST; Pfaffl 2001; Pfaffl *et al.* 2002) was used to measure the relative expression of the G6PDH gene. The relative expression ratio of a target gene is computed as:

$$\text{Ratio} = (E_{\text{target}})^{\Delta \text{CPtarget(control-sample)}} / (E_{\text{ref}})^{\Delta \text{Cpref (control-sample)}}$$

The relative expression ratio of the target gene, G6PDH, was computed based on its real-time PCR efficiencies (E) and the CP difference between an unknown sample and a control (ΔCP control-sample). G6PDH was normalized by the housekeeping gene deemed appropriate by the BestKeeper software. The three P treatment replicates were analyzed at various cDNA template concentrations (three times more, and three times less concentrated) for each sample to account for different reverse transcription efficiencies, and confirm precision and reproducibility of the analysis.

4.4 **Results**

To investigate the effects of phosphate concentration on the regulation of fungal genes during the AM fungi-plant symbiosis, a partial putative *G. intraradices* G6PDH cDNA gene sequence was obtained from Dr. P.J. Lammers (New Mexico State University). To verify that this sequence encoded part of the *G. intraradices* G6PDH gene, the nucleotide sequence was translated and compared to those of other fungi from the GenBank database using Blast. Close matches of five fungal G6PDH sequences with homology ranging from 79% to 67% were found, and these sequences were aligned with the *G. intraradices* G6PDH fragment (Fig. 1). This analysis revealed that the *G. intraradices* G6PDH partial sequence encodes an 88 amino acid segment corresponding to amino acids 408-498 of the 505 amino acid *S. cerevisiae* G6PDH protein.

To verify whether *G. intraradices* G6PDH gene expression could be assayed using QRT-PCR, it was necessary to first design and test specific PCR primers. G6PDH primers, designed using the partial sequence, were tested for the ability to PCR a G6PDH-specific amplicon from *G. intraradices* germinated-spore cDNA. The G6PDH primers yielded one band of the expected size (227-bp), which was confirmed by DNA sequencing. PCR amplification was also done to test the primers for the housekeeping control genes. The primers encoding β-tubulin and the 18S rRNA yielded single 575-bp and 382-bp amplicons, respectively, as expected (data not shown).

The BestKeeper software was used to determine which of the two HK genes to use as an internal control for QRT-PCR. The least variable HK gene was chosen to correct for sample to sample variation, and improve the reliability of the QRT-PCR. Upon comparison with an index of ten best-suited standards in the BestKeeper software, it was established

that the 18S rRNA primer set was best suited for use as an internal control in these experiments. The β-tubulin HK gene exhibited excessive variation between P treatments, with a standard deviation of 1.39, putting it above the required threshold of 1.0 to be considered consistent enough to be used as a control (Pfaffl *et al.* 2004). The 18S rRNA gene had a standard deviation of 0.87, thus making it suitable for use in further analyses with the relative expression software tool (REST) software, as an internal control to normalize the expression ratio of G6PDH.

The 18S and G6PDH primer sets amplified unique products of expected size from spore cDNA and colonized root cDNA templates in conventional PCR. Prior to initiating the QRT-PCR, all RNA samples were tested by conventional PCR for possible DNA contamination. No amplified products were detected for either primer set (data not shown). Included with all QRT-PCR runs were negative controls consisting of uncolonized transformed carrot root cDNA template and RT-PCR reaction mixtures excluding cDNA template. Dissociation curve analysis was used to show the specificity of each primer set resulting in single amplified products (Fig. 2 A, B). The resulting specific melting temperatures were: G6PDH, 78.8 ± 0.5°C (Experiment 1) and 78.6 ± 0.2°C (Experiment 2), and 18S, 82.8 ± 0.7°C (Experiment 1) and 82.9 ± 0.3°C (Experiment 2).

Standard curves of real-time QRT-PCR efficiencies were generated for *G. intraradices* transcripts for transformed root cultures subjected to various P concentrations for 10 weeks (Fig. 2 E, F), or exposed to additional P for two-hours (data not shown). Serial dilutions of RT-PCR products for each gene, in the range of 10^{-1} to 10^{-9}, were amplified by the QRT-PCR assay. The crossing points were determined at a fixed fluorescence of 0.05. Amplification plots were highly reproducible between triplicate

samples and fluorescence data from negative controls always remained below the threshold level (Fig. 2 C, D). A linear regression relationship was established between the initial concentration (ng) and the Ct of 4-5 serial dilutions, for the 18S rRNA and G6PDH amplicons. The square regression correlation coefficients (R^2) of detection, measuring the accuracy of the analysis as a quantification method, ranged between 0.996 and 0.999 for all experiments.

Through the use of the REST software, the CPs were used to measure the expression of G6PDH gene transcripts when symbiotic root cultures were grown under the various P conditions. In Experiment 1, the symbiotic root cultures were grown in three external P concentrations: LP, HP and EP. *G. intraradices* G6PDH expression in root cultures grown under HP conditions was 3.33-fold lower than the expression observed under control LP conditions (P=0.017), while G6PDH expression in the EP treatment was found to be not significant compared to the LP treatment (P=0.231) (Table 1). The significant down-regulation of G6PDH expression when colonized transformed root cultures are grown under a HP treatment, suggests that increased external P treatments have a negative effect on C metabolism of the fungus located within the host cells. No significant difference in G6PDH expression between the HP treatment and the EP treatment was observed.

The observation that G6PDH expression may be repressed in response to increased external P raised the question of how soon after exposure this response occurs. To address this question, in Experiment 2, *G. intraradices*-colonized transformed carrot roots were exposed to three treatments: No P, LP, and HP. In this experiment, the cultures were harvested after a two-hour incubation period. G6PDH expression in neither the additional

LP (P=0.734) nor the additional HP treatments (P=0.755) was significantly different from that under the control (No P).

4.5 Discussion

AM fungi exposed to high concentrations of soil P can be inhibited with respect to AM colonization of plant roots, sporulation, and promotion of plant productivity (Abbott *et al.* 1984; Lui *et al.* 2000; Trimble and Knowles 1994) compared to AM fungi exposed to low soil P concentrations. We hypothesized that these effects may, in part, be mediated through altered fungal C metabolism. This study was carried out to investigate the connection between the C and P metabolism in AM symbiosis. Although only a limited number of *G. intraradices* gene sequences are currently known, a partial G6PDH cDNA sequence was available (Lammers pers comm). G6PDH plays a role in the metabolism of both C and P, and, as such, the G6PDH gene was chosen for this study to address regulation in a C metabolic pathway as a response to external P concentrations during the AM fungal-plant symbiosis.

When colonized root cultures were grown under various P conditions for 10 weeks, there was a significant down-regulation of G6PDH expression under HP conditions as compared with LP. The effect was not significant in the response of cultures grown under EP conditions when compared to LP. As P is an essential nutrient for energy production and root growth, HP concentrations could lead to an increase in the rate of root growth resulting in less available C for the AM fungus. Therefore, the significant reduction in G6PDH expression in AM fungi exposed to HP could reflect either a host-mediated effect, a direct P effect, or a combination of both, an issue that could be resolved in future studies. These results seem to agree with those of Olsson *et al.* (2002) who reported a reduction of C translocation to the fungus with increasing P. Other studies have also shown effects on fungal metabolism in response to host metabolic conditions (Shachar-Hill *et al.* 1995;

Bücking and Shachar-Hill 2005). This type of regulatory mechanism may enable the host to protect itself from a potential parasitic relationship with the fungal partner under conditions in which the host does not require P from the fungus. Alternatively, the fungus may have evolved a mechanism to directly detect P and thus anticipate C limitation.

The question of how quickly this regulatory mechanism responds to changing external P concentrations remains unsolved. Ezawa *et al.* (2003) observed that a maximum polyP accumulation in ERM was attained less than three hours following P application to the soil. Kojima and Saito (2004) observed that the rate of P efflux from excised IRM decreased with time when in the presence of glucose, and data in their study were recorded between 0-2 hours. This result influenced the design of Experiment 2, in which a 2 hour incubation period was used to investigate whether G6PDH gene expression levels were affected by short-term exposure to additional P. If the response to increased external P concentrations were to occur that quickly, it seemed reasonable to quantify G6PDH expression after a similarly short interval.

No significant differences in G6PDH expression levels were observed among the additional P treatments when compared with the control condition. While polyP efflux has been shown in the presence of glucose (Kojima and Saito 2004) with rates comparable to the amount of phosphate reduction in the IRM (Solaiman and Saito 2000), there was no significant effect on G6PDH when an intact symbiotic root system was exposed to increased P concentrations in a two-hour time interval. If, in fact, the activity of G6PDH is linked to C flow from the host, these results may suggest that the metabolic processes involving C metabolism of the host are not rapid. Further studies, however, are needed with increased time intervals to confirm such an observation.

The successful development of *G. intraradices*-specific primers for G6PDH enabled us to perform QRT-PCR to examine P effects on the carbon metabolism. The down regulation of *G. intraradices* G6PDH expression under a high P treatment may suggest that C translocation from the host to the fungal partner is affected. The expression of G6PDH is not affected by increased P applications in a short incubation period, although maximum polyP accumulation occurs within three hours, and phosphate efflux decreases over time when glucose is present. This may suggest that neither P nor C flow from the host affect the fungal symbiont in the short-term (less than two hours).

This study is only an initial step in the examination of the regulation of genes of AM fungal C metabolism in response to external P conditions. More HK genes should be identified, and examined for their variability under differing P concentrations. Further analyses, studying C regulation under various external P conditions, and the expression of other genes encoding enzymes of C metabolic pathways, are needed to determine if the host is regulating C flow to the symbiont in response to external P treatments. To our knowledge, however, this is the first report of the quantification of G6PDH gene expression in the AM fungus *G. intraradices* by real-time QRT-PCR.

Table 4.1. The effect on AM fungal G6PDH gene expression when grown under various P concentrations. Values indicated by ns were deemed not significant when compared to the control P treatment. A negative value indicates down-regulation in gene expression.

Control P Treatment	G6PDH regulation under HP concentration	G6PDH regulation under EP concentration
LP	-3.33	ns
HP	ns	ns

5'--

CAATCGAAGATTTTCAGATTTGAAGATTCCCGATGCGTATGAAGCAT

TGATACTTGATGTGTTGAAAGGTGATCATTCCAATTTTGTGAGAGAC

GATGAATTAGATGCCGCCTGGAAGATTTTTACACCACTTTTACATAA

AATTGATAATGAAAAATTGCTCCTCAACCTTACGCATACGGTACACG

TGGTCCACCTGGTATTGAAGAGTTTGTATCTAAATATGGATTCAAGA

GGCCGACACAAGAATATAATTGGCCGGAACATAAGATTAACAAGCTT

TAAATGATCCATCTTCATCAGCCAATTGTTATATTGTATTTTATTGTT

AATTTCTTTTTTGTCCTTTTTTTAACGACATGGGTTTCTCTGTATATA

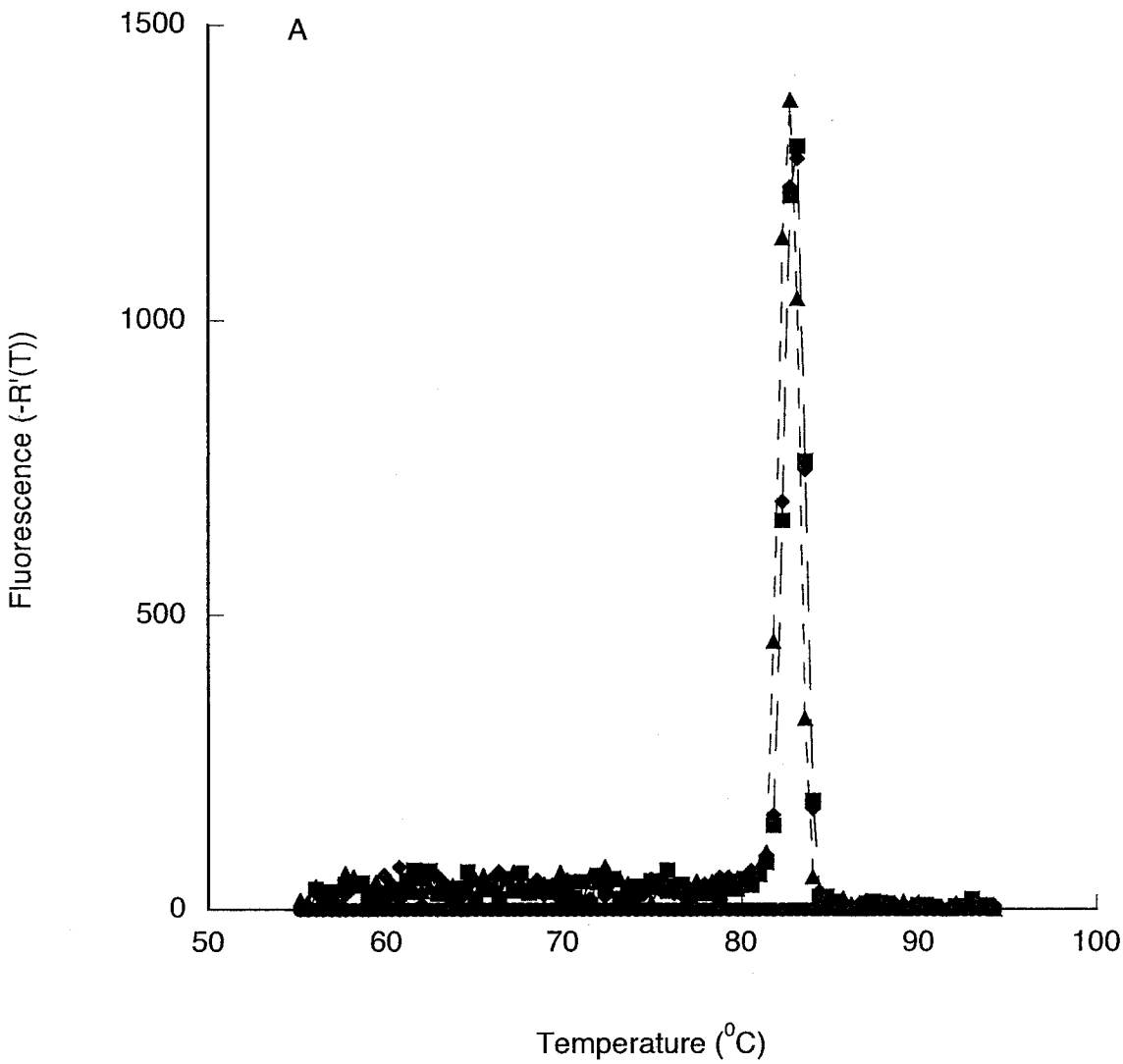
AGTTTTGTAGCAAGGGAAAATTGTTAATATAATGTAGTCTATGAATTT

TGATTGCTTGTATTTGCGCTTGTTCTTTTCTTGAATTATTATATGTAA

ATTTTCATGATAATTCATTAAATTCGTCTTTTAACTAAAAA--3'

Fig. 4.1. The 515-bp partial nucleotide sequence of the *G.intraradices* G6PDH. G6PDH-LS1 and G6PDH-rev primers are underlined.

Fig. 4.1. Multiple alignment of the partial *G.intraradices* G6PDH deduced amino acid sequence with homologous regions of G6PDHs from the following fungi: *Magnaporthe grisea*, *Emericella nidulans*, *Neurospora crassa*, *Saccharomyces cerevisiae*, and *Aspergillus niger*. The degree of homology among the various sequences at each residue is indicated as complete conservation (*), moderate conservation (.), or little or no homology (unmarked).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70
80
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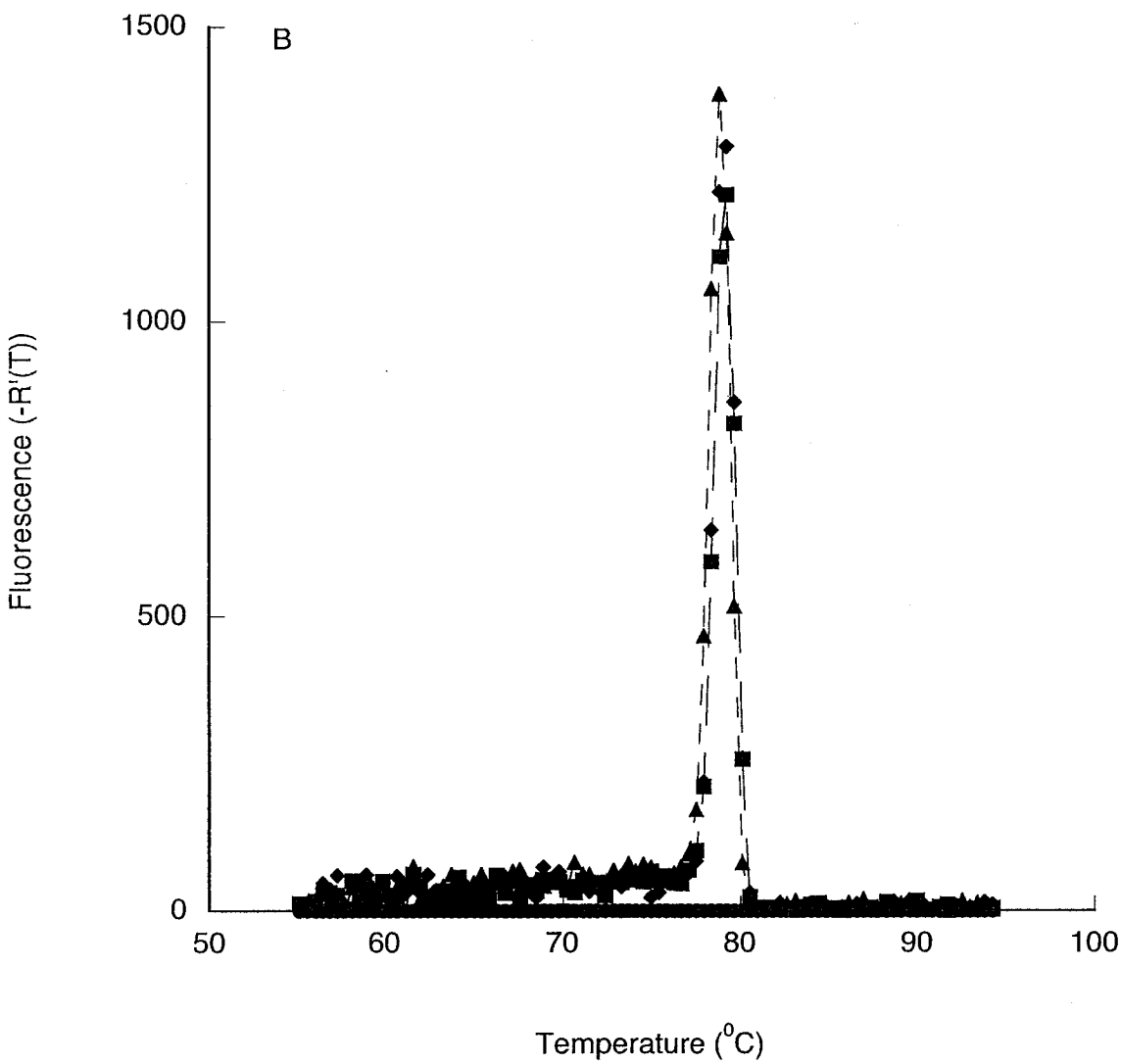
100
Temperature (°C)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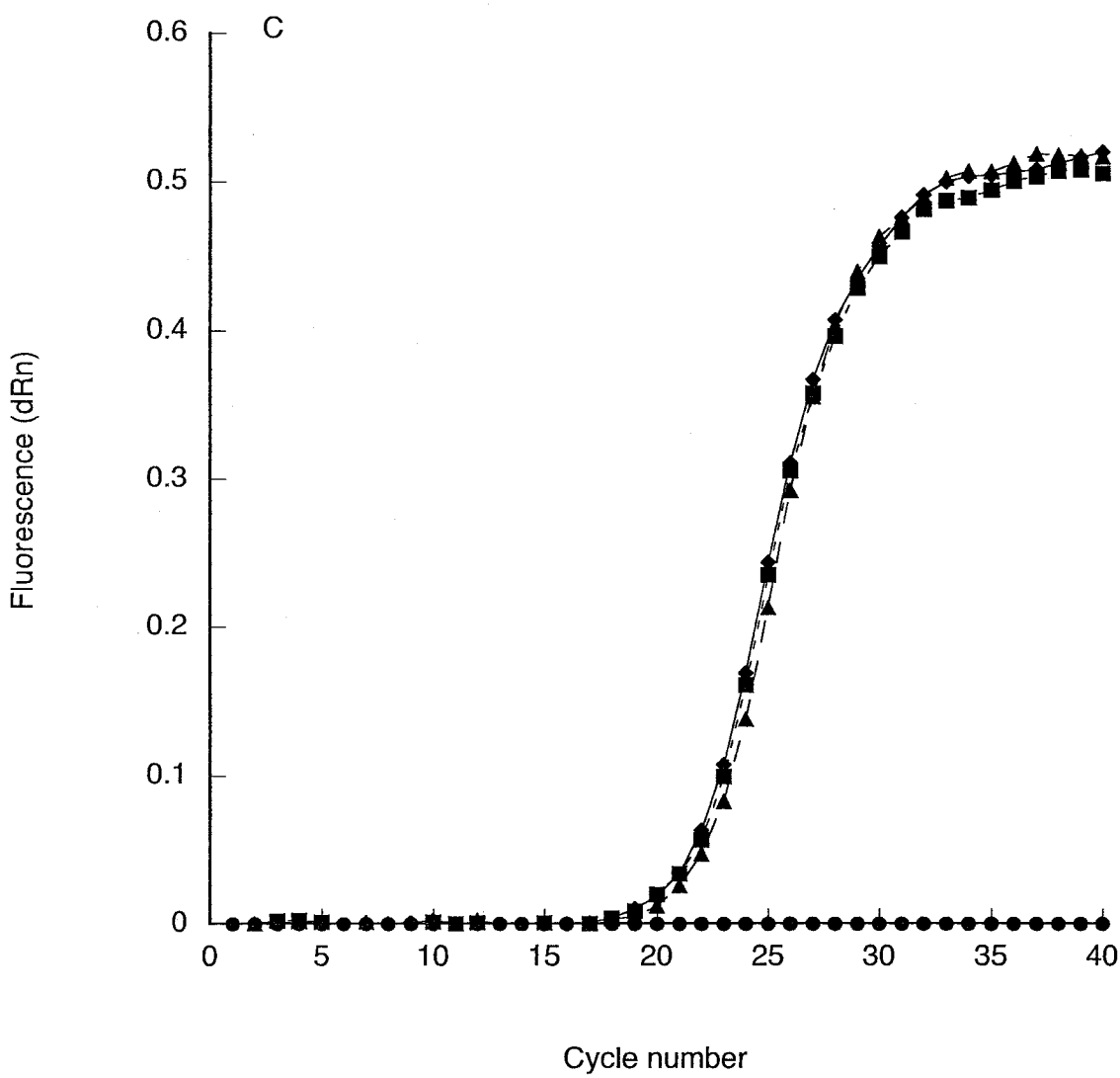
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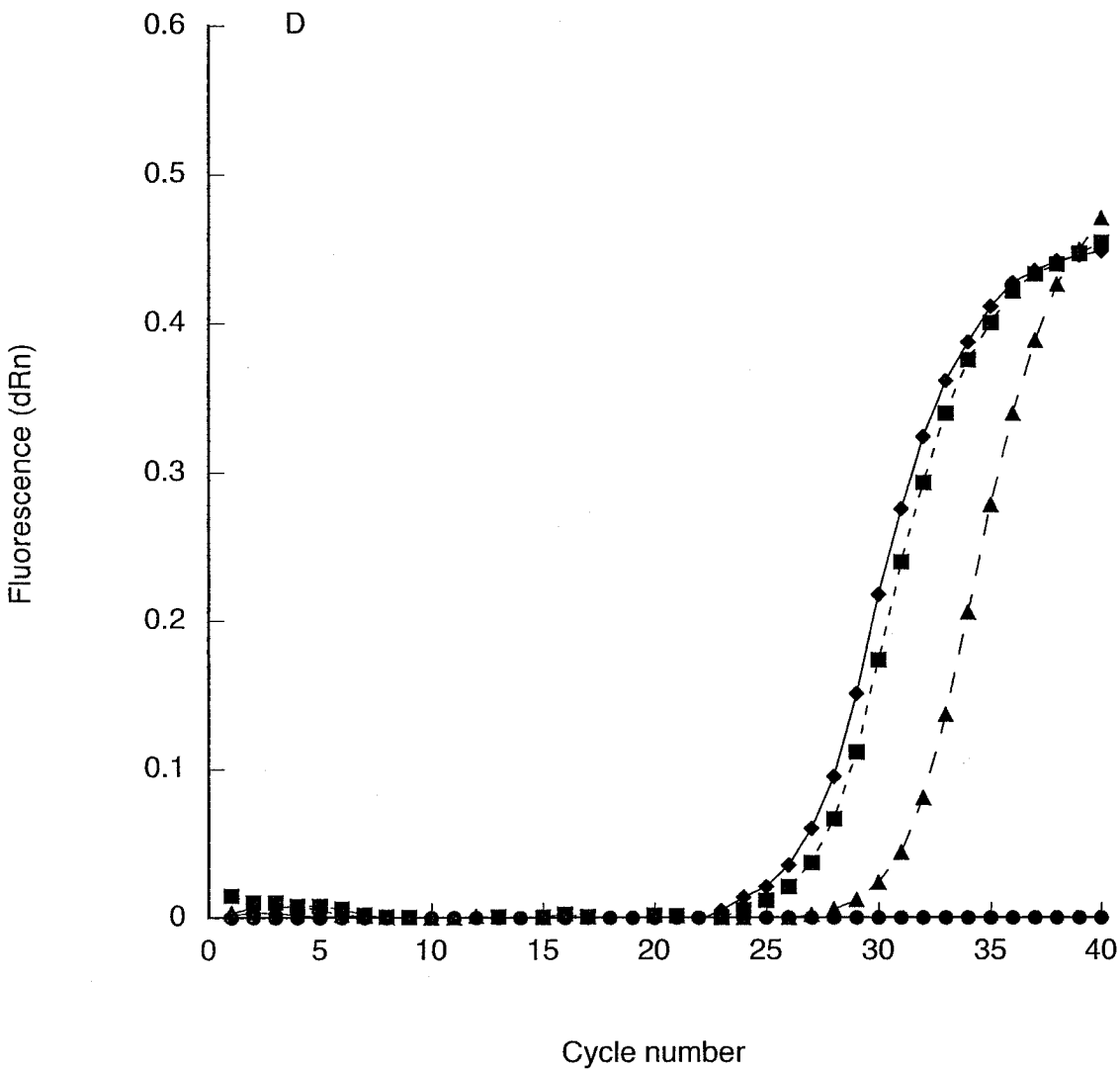
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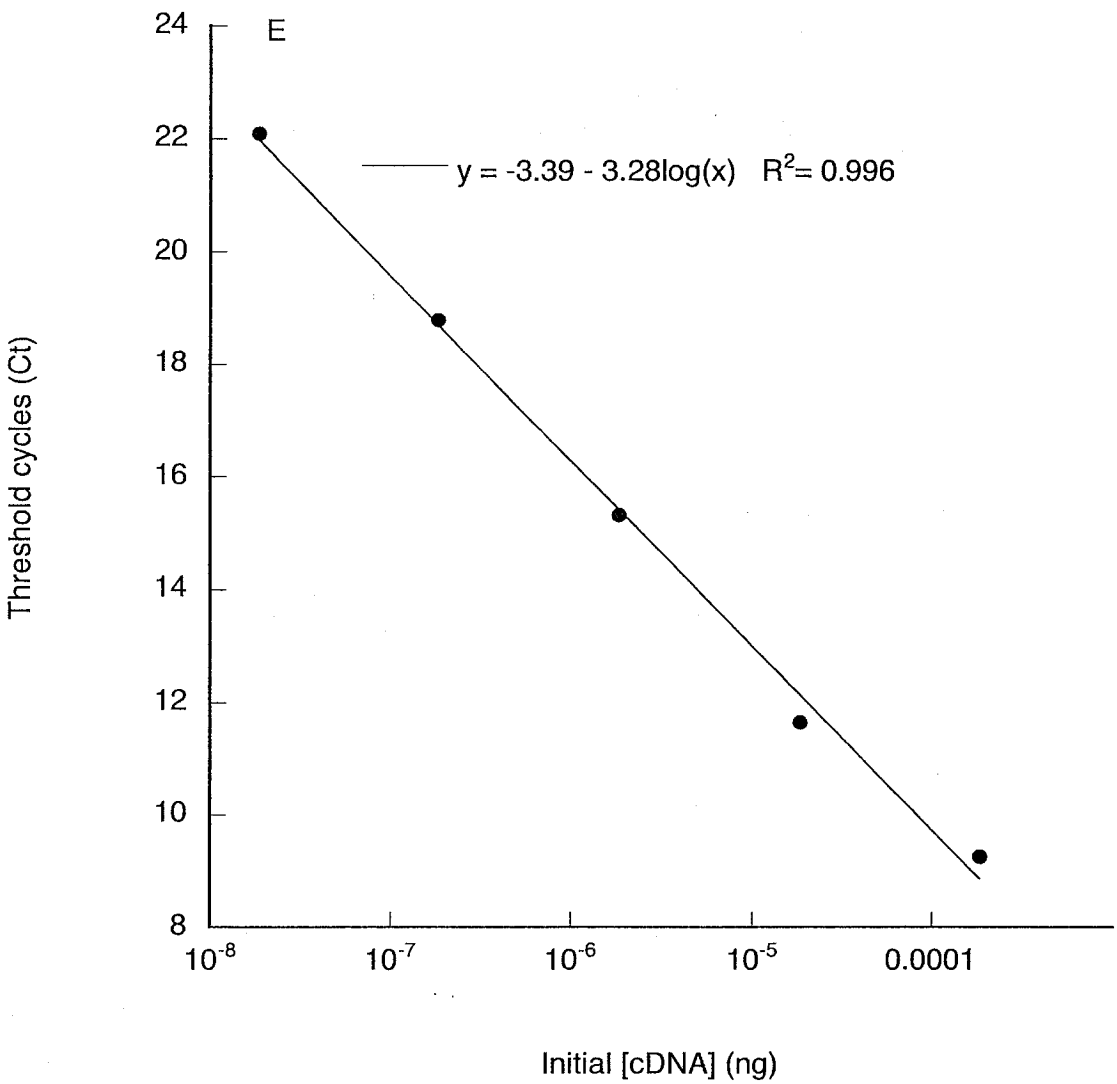
E
y = -3.39 - 3.28log(x) R2= 0.996
Threshold cycles (Ct)
24
22
20
18
16
14
12
10
8
10-8
10-7
10-6
10-5
0.0001
Initial [cDNA] (ng)

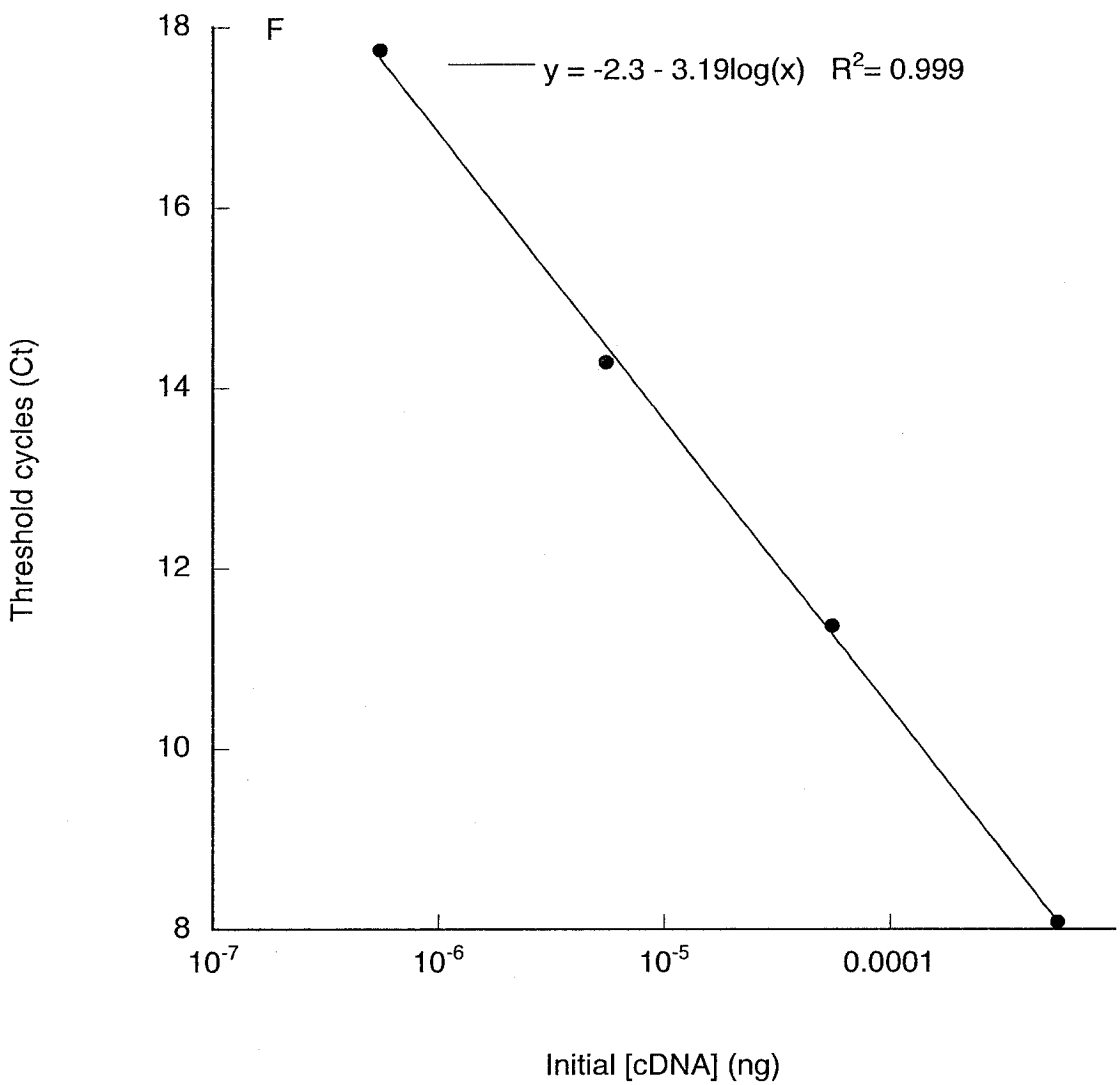
F
y = -2.3 - 3.19log(x) R2= 0.999
18
16
14
12
10
8
Threshold cycles (Ct)
10-7
10-6
10-5
0.0001
Initial [cDNA] (ng)

Fig. 4.2. Examples of: dissociation curves for the real-time QRT-PCR products amplified for experiment 1 (**A**, 18S rRNA; **B**, G6PDH); kinetics of fluorescence signal versus cycle numbers measured during amplification of the *G. intraradices* IRM gene transcripts for transformed roots cultures subjected to various P concentrations for 10 weeks (**C**, 18S rRNA; **D**, G6PDH); standard curves of real-time QRT-PCR efficiencies for *G. intraradices* IRM gene transcripts for transformed root cultures subjected to various P concentrations for 10 weeks (**E**, 18S rRNA; **F**, G6PDH). Symbols for A-D: υ, EP; ν, LP; σ, HP; -, uncolonized roots; λ, negative control.

Chapter 5. General Discussion and Conclusions

Inoculation of commercially-grown strawberry cultivars with different AM fungi resulted had variable effects on daughter plant production, and increases in total fruit yield through cultivar-AM species interactions. While the number of daughter plants produced per mother increased by 50% in cultivar Glooscap inoculated with *G. intraradices*, the number generally declined with inoculation for other cultivar-AM species combinations. These results are consistent with other studies that reported *G. intraradices* and other single-species inocula improved plant productivity (de Silva *et al.* 1996; Khanizadeh *et al.* 1995; Kiernan *et al.* 1984; Chavez and Ferreo-Cerrato 1990). Under conditions of high soil fertility, we found that inoculation with *G. intraradices* alone was more effective at increasing daughter plant production than inoculation with mixed *Glomus* species.

Fruit production was enhanced by AM inoculation for all cultivars during the first harvest year. The combination of Kent inoculated with *G. intraradices* produced the highest yield, while yields for the treatment combinations of Kent and Glooscap inoculated with *G. mosseae* were not significantly different ($P<0.05$). These results are consistent with the observations of Chavez and Ferrera-Cerrato (1990) in which total fruit yield was increased as a result of cultivar-AM interactions. The excessive P level in our experiment of >400 $kg \cdot ha^{-1}$ significantly exceeds the 150 $kg \cdot ha^{-1}$ fertilizer limit in which Sharma and Adholya (2004) observed increases in strawberry yield and berry mass in response to AM inoculation. The cultivar-AM species interaction effects indicate strawberry plant

cultivars exhibit MD among cultivars, as shown in other plant species (Linderman and Davies 2004; Koide *et al.* 1998; Khalil *et al.* 1994).

The lack of effect by the host and non-host nature of the rotation crops on the soil mycorrhizal potential, mother plant productivity, or daughter plant mycorrhizal development, suggests that under high soil fertility conditions crop inoculation may be the only option for management of the symbiosis.

The low mycorrhizal potential of the soils in these field studies (<5%) maybe due to the high soil fertility, in particular P. P uptake by AM fungi colonizing plant roots is regulated by the host through C flow (Shachar-Hill *et al.* 1995; Bücking and Shachar-Hill 2005). In our study, the reduction in G6PDH expression when colonized transformed carrot roots were grown under HP conditions compared to LP, suggests a reduction in the flow of C supplied by the host root. Such decreases could result in reduced fungal growth and root colonization (Bethlenfalvay *et al.* 1983; Amijee *et al.* 1989), leading to lower levels of inocula in the soil (i.e. the mycorrhizal potential), as seen in our field studies. The high soil P levels (>400 $kg \cdot ha^{-1}$) for both of our field experiments may have contributed to the low root colonization levels of the strawberry plants (<16%) through reduced C flow regulated by the host.

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5. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Job Title/Classification	Trained in the safe use of biological safety cabinets within the last 3 years? If yes indicate training date.
Jean-Francois Lauzon	Natural Resource Scienc	Graduate student	
David Meek	Natural Resource Scienc	Technician	
Lynda Stewart	Natural Resource Scienc	Graduate student	
Frederic D'Aoust	Natural Resource Scienc	Graduate student	

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues, toxins of biological origin) & designated biosafety risk group

Bacteria from the genus Sinorhizobium, as well as other related bacteria (Rhizobia), are nitrogen-fixing symbionts of legume plants. These bacteria were previously isolated from healthy plants and none are known pathogens. We will also utilize standard (non-pathogenic) strains of Escherichia coli and bacteriophage specific to Sinorhizobium meliloti and E. coli for molecular genetic and cloning experiments.

ii) the procedures involving biohazards

DNA extraction and cloning from Rhizobia. These strains will also be grown with plant hosts in plant growth chambers to test symbiotic nitrogen fixation. We will be growing these strains and bacteriophage in liquid culture and on agar plates. Most of the work will involve molecular biological techniques using DNA maintained in plasmids in an E. coli background. We will be using standard bacterial genetics protocols such as conjugation, transformation and transduction.

iii) the protocol for decontaminating spills

Spills will be mopped up with paper towels, surfaces will be sterilized using ethanol or dettol, and contaminated material will be autoclaved (contaminated paper in biohazard bags) and discarded.

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) that could increase the hazards?

No

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

Yes

9. What precautions will be taken to reduce production of infectious droplets and aerosols?

Use of pipette guns rather than glass pipettes, regular maintenance of pipette guns, use of disposable tips, use of capped tubes, training in asceptic technique using bunsen burner. Any pathogen-containing material can be manipulated in a vertical flow hood located in Dr. Niven's lab (MS3-050).

10. Will the biohazardous materials in this project expose members of the research team to any risks that might require special training, vaccination or other protective measures? If yes, please explain.

No

11. Will this project produce combined hazardous waste – i.e. radioactive biohazardous waste, biohazardous animal carcasses contaminated with toxic chemicals, etc.? If yes, please explain how disposal will be handled.

Np

12. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
Macdonald Stewart	MS3-050	Canadian cabinets	BM6 2A	7079	Sept 2004